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In re Application of:  
Akira ASAKURA, et al.

Serial No.: 09/712,768

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For: **CYTOCHROME C OXIDASE  
ENZYME COMPLEX**



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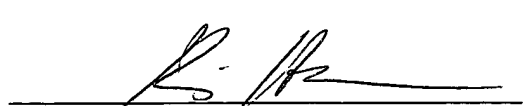
Enclosed please find a certified copy of the following priority document:

1. Appln. No. 99122842.0, filed November 17, 1999, in Europe.

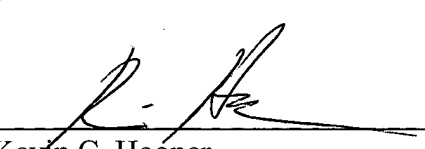
Applicants claim priority based on the aforesaid application under the provisions

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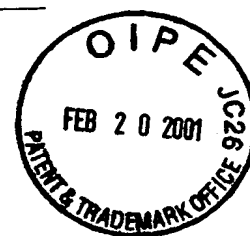
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99122842.0

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17. Nov. 1999

Cytochrome c oxidase enzyme complex

The present invention relates to recombinant production of 2-keto-L-gulonic acid and biological materials useful thereof.

5        Cytochrome c oxidase (cytochrome aa<sub>3</sub>; EC 1.9.3.1) is a terminal oxidase enzyme in the aerobic respiratory electron transport system of mitochondria and many bacteria. The enzyme is a cytoplasmic membrane spanning complex and catalyzes the final electron excreting; re-oxidation of ferrocytochrome c (electron donor) at periplasmic surface / reduction of molecular oxygen (electron acceptor) to water at cytoplasmic surface. The  
10        reaction couples with extrusion of protons across the membrane and this coupling is indispensable for biological energy conservation from substrate oxidation.

Various types of cytochrome complex, e.g. aa<sub>3</sub>, a<sub>1</sub>, caa<sub>3</sub>, o, bo, co, bd-types, have been identified as functional terminal oxidases; purification and characterization of some terminal oxidases have been reported. Matsushita et al. reported that *Acetobacter aceti*  
15        IFO 3283 contains two terminal oxidases, cytochrome a<sub>1</sub> and o, and purified and characterized the cytochrome a<sub>1</sub> (Proc. Natl. Acad. Sci., USA, 87: 9863, 1990; J. Bacteriol. 174: 122, 1992). Matsushita et al. also reported the purification of cytochrome o from *Gluconobacter* (Biochem. Biophys. Acta, 894: 304, 1987). Tayama et al. disclosed a terminal oxidase (cytochrome a<sub>1</sub>) genes of *A. aceti* (JP 93-317054); they purified the  
20        oxidase enzyme which consisted of four subunits of 72, 34, 21, and 13 kDa and contained heme a and heme b. The oxidases in *Acetobacter* and *Gluconobacter* belong to quinol oxidase. Cytochrome aa<sub>3</sub> (cytochrome c oxidase) has been purified from bovine heart, yeast, and many bacteria including *Paracoccus denitrificans* (Solioz et al., J. Biol. Chem., 257: 1579-1582, 1982) and *Rhodobacter sphaeroides* (Hosler et al., J. Biol. Chem., 267:  
25        24264-24272, 1992).

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Mammalian (mitochondrial) cytochrome c oxidase (aa3-type) complex contains 13 different subunits; the three core subunits I, II and III (CO I, II and III) are encoded by the mitochondrial DNA, while the remaining 10 are by nuclear origin. Bacterial aa3-type cytochrome c oxidase also contains the three core subunits, which are homologous to the  
5 mitochondrial core subunits. However, it is reported that CO III was easily lost during purification, resulting in preparations composed of CO I and CO II only (Ludwig et al., Proc. Natl. Acad. Sci. USA, 77: 196-200, 1980). The cytochrome c oxidase complex consisting of the two-subunit (CO I and II) showed redox activity with generation of electrochemical proton gradient. In case of *P. denitrificans* (Haltia et al., The EMBO  
10 Journal, 10: 2015-2021, 1991) and *R. sphaeroides* (Cao et al., Gene, 101: 133-137, 1991), both two-subunit-type (CO I / II) and three-subunit-type (CO I / II / III) of complexes were isolated by different purification methods. Genetically, genes for CO II and III are located in a operon, while gene for CO I is independently located (Raitio et al., The EMBO Journal, 9: 2825-2833, 1987, Shapleigh et al., Proc. Natl. Acad. Sci. USA, 89: 4786-4790,  
15 1992).

Terminal oxidases described above play an important role in growth under aerobic condition to accomplish reduction of molecular oxygen. In oxidative fermentation, respiratory chain including terminal oxidase works for completing oxidation of a substrate to produce an oxidized product. In this context, it is very important to improve the  
20 efficiency of the respiratory chain for efficient oxidative fermentation.

*G. oxydans* DSM 4025 produces 2-keto-L-gulonic acid (2KGA), an important intermediate in a process of L-ascorbic acid production, from L-sorbose via L-sorbose (T. Hoshino et al., European Patent Application No. 88116156 A2). The oxidation of the substrate, L-sorbose, to 2KGA was considered to be completed by the respiratory electron  
25 transport chain. The terminal oxidase which catalyzes the final electron excreting to oxygen might be one of the kinetic rate-limiting steps in the 2KGA production system as well as other redox components. The primary dehydrogenase responsible for 2KGA formation from L-sorbose was isolated (T. Hoshino et al., European Patent Publication No. 606621) and the genes were cloned and sequenced; four isozymes were found (T.  
30 Hoshino et al., European Patent Publication No. 832974 A2) and their direct electron acceptor, cytochrome c551, was purified and its gene was cloned (T. Hoshino et al., European Patent Application No. 97105583.5). The terminal oxidase was, however, not isolated and its genes were not cloned.

The present invention was aimed for providing the materials for improving the  
35 quantity and quality of cytochrome c oxidase and improving oxidative fermentation completed by the cytochrome c oxidase with the aid of making novel cytochrome c oxidase genes available. The microorganism deposited as *Gluconobacter oxydans* under the



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accession No. DSM 4025 is a preferable example of a source which provides the novel cytochrome c oxidase and its genetic materials of the present invention.

The present invention provides a novel cytochrome c oxidase enzyme complex which are isolated from natural source or prepared with the aid of genetic engineering.

5 Such an enzyme complex having cytochrome c oxidase activity are obtainable or obtained from biological or genetic material originated from a microorganism identified as *G. oxydans* DSM 4025 or a biologically and/or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of *G. oxydans* DSM 4025. Thus the present invention provides a novel cytochrome c oxidase complex which is useful as an  
10 essential component mediating electron transfer in the respiratory chain.

A cytochrome c oxidase complex exemplified herein is the one which shows the following physicochemical properties of: (i) showing the presence of at least two core subunits of I (COI) and II (COII) wherein; apparent molecular mass of COI is about 43 +/- 10 kDa by SDS-PAGE analysis; and apparent molecular mass of COII is about 36 +/-  
15 10 kDa by SDS-PAGE analysis, and (ii) absorption spectrum showing aa3-type cytochrome c oxidase being; 605 +/- 1 nm peak in reduced minus oxidized difference spectrum. Such a cytochrome c oxidase complex can be provided as a substantially homogeneous isolate derived from a culture of a microorganism identified as *G. oxydans* DSM 4025 or biologically and/or taxonomically homogeneous cultures of a  
20 microorganism having the identifying characteristics of *G. oxydans* DSM 4025.

A novel cytochrome c oxidase complex of the present invention can be also provided in the form of a recombinant enzyme, which may comprise a recombinant polypeptide as a core subunit I (COI), wherein the recombinant polypeptide can be selected from the group consisting of polypeptides having an amino acid sequence identified by SEQ ID NO:  
25 2 and those having amino acid sequences having 85% or higher identity with the said sequence and being capable of providing the complex with cytochrome c oxidase activity. Further, the other core subunit II (COII) and III (COIII) can be recombinant polypeptide(s) selected from the group consisting of those containing amino acid sequences identified by SEQ ID NOs: 4, 6 and/or 8 and those containing amino acid  
30 sequences having 85% or higher identity with any one of the said amino acid sequences, and is (are) capable of providing the complex with cytochrome c oxidase activity.

As another aspect of the present invention, the respective core subunits, i.e. COI, COII and COIII are provided as recombinant polypeptides which are useful for the components of the novel cytochrome c oxidase complex of the present invention.

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Exemplified herein as COI is a recombinant polypeptide which is involved in cytochrome c oxidase complex, which polypeptide comprises an amino acid sequence identified by SEQ ID NO: 2 or an amino acid sequence having 85 % or higher identity with the said amino acid sequence and is capable of providing the complex with cytochrome c oxidase activity. A recombinant COI may be a polypeptide capable of providing the complex of the present invention with cytochrome c oxidase activity, which is encoded by a recombinant DNA fragment comprising a DNA sequence selected from the group consisting of:

- (a) the DNA sequence identified by SEQ ID NO: 1, and
- 10 (b) the DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 2 or amino acid sequences having 85 % or higher identity with the said amino acid sequence.

Also exemplified herein as COII is a recombinant polypeptide which is involved in cytochrome c oxidase complex of the present invention, which polypeptide contains an amino acid sequence identified by SEQ ID NO: 4, or an amino acid sequence having 85 % or higher identity with the said amino acid sequence and is capable of providing the said complex with cytochrome c oxidase activity. A recombinant COII may be a polypeptide capable of providing the complex of the present invention with cytochrome c oxidase activity, which is encoded by a recombinant DNA fragment containing a DNA sequence selected from the group consisting of:

- (a) the DNA sequence identified by SEQ ID NO: 3, and
- (b) the DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 4 or amino acid sequences having 85 % or higher identity with the said amino acid sequence.
- 25

Moreover, also exemplified herein as COIII is a recombinant polypeptide which is involved in cytochrome c oxidase complex of the present invention. Such recombinant polypeptide contains either or both of the amino acid sequences identified by SEQ ID NOs: 6 and 8, respectively or amino acid sequences having 85 % or higher identity with the respective amino acid sequences of SEQ ID NOs: 6 and 8 and is capable of providing the said complex with cytochrome c oxidase activity. A recombinant COIII may be a recombinant polypeptide capable of providing the complex of the present invention with cytochrome c oxidase activity, which is encoded by a recombinant DNA fragment containing one or more DNA sequence(s) selected from the group consisting of:

- 35 (a) the DNA sequence identified by SEQ ID NO: 5,
- (b) the DNA sequence identified by SEQ ID NO: 7,
- (c) the DNA sequences which encode polypeptides having an amino acid sequence

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identified by SEQ ID NO: 6 or amino acid sequences having 85 % or higher identity with the said amino acid sequence, and

- (d) the DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 8 or amino acid sequences having 85 % or higher identity with the said amino acid sequence.

In further aspect of the present invention, recombinant DNA fragments which are useful for preparing the respective core subunits, i.e. COI, COII and COIII by genetic engineering are provided. Such recombinant polypeptides are useful for the components to be involved in the novel cytochrome c oxidase complex of the present invention. As explained above, these polypeptides should be capable of providing the cytochrome c oxidase complex of the present invention with cytochrome c oxidase activity

Exemplified herein as a recombinant DNA fragment for COI is a DNA fragment which encodes a polypeptide involved in cytochrome c oxidase complex and comprises a DNA sequence selected from the group consisting of:

- (a) the DNA sequence identified by SEQ ID NO: 1, and  
(b) the DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 2 or amino acid sequences having 85 % or higher identity with the said amino acid sequence.

Also exemplified herein as a recombinant DNA fragment for COII is a DNA fragment which encodes a polypeptide involved in cytochrome c oxidase complex and contains a DNA sequence selected from the group consisting of:

- (a) the DNA sequence identified by SEQ ID NO: 3, and  
(b) the DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 4 or amino acid sequences having 85 % or higher identity with the said amino acid sequence.

Moreover, also exemplified herein as a recombinant DNA fragment for COIII is a DNA fragment which encodes a polypeptide involved in cytochrome c oxidase complex and contains one or more DNA sequence(s) selected from the group consisting of:

- (a) the DNA sequence identified by SEQ ID NO: 5,  
(b) the DNA sequence identified by SEQ ID NO: 7,  
(c) the DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 6 or amino acid sequences having 85 % or higher identity with the said amino acid sequence, and  
(d) the DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 8 or amino acid sequences having 85 % or higher identity with the said amino acid sequence.

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Moreover, in another aspect of this invention, it is provided an expression vector comprising one or more of the above mentioned recombinant DNA fragments, which vector is suitable for the expression in an organism, pro- or eukaryotic host cell.

Further, it is another aspect of the present invention to provide a recombinant organism which is introduced with an expression vector mentioned above. Such a recombinant organism of the invention will be useful for the genetic preparation of a recombinant cytochrome c oxidase complex of the present invention and also applicable to a process for producing 2KGA from L-sorbose or D-sorbitol in an appropriate culture medium. Host cells for the recombinant organism of the present invention may be of eukaryotic origin, preferably a mammalian or plant cell, or may be of prokaryotic origin. These host cells may in particular be obtained from bacteria, preferably *G. oxydans* DSM No. 4025 and biologically and/or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of *Gluconobacter oxydans* DSM 4025.

This invention is also directed to a process for producing cytochrome c oxidase, which comprises cultivating the recombinant organism of this invention mentioned above, particularly the recombinant organism containing a preferred DNA sequence exemplified herein, in an appropriate culture medium and recovering the cytochrome c oxidase from the culture medium.

Further, this invention is also directed to a process for producing 2KGA from L-sorbose or D-sorbitol which comprises cultivating a recombinant organism of the present invention as mentioned above in an appropriate culture medium and recovering 2KGA from the culture.

The following figures are included to further illustrate the present invention together with the detailed description given below.

Figure 1 shows absorption spectra pattern of aa3-type cytochrome c oxidase of *G. oxydans* DSM4025. Spectra were recorded at room temperature at a protein concentration of 0.08 mg/ml in 25 mM Na-HEPES (pH 7.5) containing 0.5% sucrose monolaurate and 5% glycerol. [A] shows the spectrum of the oxidized form. [B] shows the spectrum of the reduced form. [C] shows reduced minus oxidized difference spectrum.

Figure 2 shows SDS-PAGE analysis of the purified cytochrome c oxidase aa3 of *G. oxydans* DSM4025. The purified enzyme (at 0.5 mg/ml of protein concentration) was

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denatured by incubation with 2% SDS, 50 mM dithioerythritol, 62.5 mM Tris-HCl (pH 6.8) and 10% glycerol at 37°C for 5 hours. Electrophoresis was carried out at 12.5% acrylamide concentration according to the method of Laemmli (Nature, 227: 680-685, 1970) with the buffer consisting of 25 mM Tris, 0.192 M glycine, and 0.1% SDS. A and B  
5 were 6 and 3 mg of the purified enzyme, respectively. C was low range prestained SDS-PAGE standards (Bio-Rad Laboratories, CA U.S.A.).

Figure 3 shows an alignment of the partial amino acid sequences of CO I from *G. oxydans* DSM4025 with ones from other organisms.

Figure 4 shows an alignment of the partial amino acid sequences of CO II from *G. oxydans* DSM4025 with ones from other organisms.  
10

Figure 5 shows an alignment of the partial amino acid sequences of CO III from *G. oxydans* DSM4025 with ones from other organisms.

Figure 6 shows primers for PCR amplification of the partial CO I, II and III genes of the cytochrome c oxidase complex from *G. oxydans* DSM4025.

Figure 7 shows the physical maps of the 8.0 kb PstI and 9.3 kb EcoRI fragments containing "CO I" and "CO II and III" genes, respectively.  
15

Figure 8 shows an alignment of the complete amino acid sequence of the CO I subunit from *G. oxydans* DSM4025 with ones from other organisms.

Figure 9 shows a genetic map of pVKcoxes used for expressing the genes of cytochrome c oxidase complex of *G. oxydans* DSM4025.  
20

The novel cytochrome c oxidase complex of the present invention belongs to a family of proteins which function as a terminal oxidase and its genes. More particularly, the novel cytochrome c oxidase of the present invention is useful as a terminal oxidase oxidizing cytochrome c, an electron acceptor for dehydrogenases such as alcohol and  
25 aldehyde dehydrogenase (AADH) and thus is useful as essential component mediating electron transfer in the respiratory chain. A cytochrome c oxidase complex of the present invention may be those isolated from natural source or prepared with the aid of genetic engineering. Such an enzyme complex having cytochrome c oxidase activity is obtainable from biological material originated from a microorganism identified as *G. oxydans* DSM  
30 4025 or a biologically and/or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of *G. oxydans* DSM 4025. The cytochrome c oxidase complex of the present invention shows the following physico-chemical characteristics: the complex shows an absorption of aa3-type cytochrome c oxidase in reduced minus oxidized

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difference spectrum, a peak at 605 +/- 1 nm; two polypeptides involved in the cytochrome c oxidase complex have apparent molecular masses of about 43 +/- 10 kDa and 36 +/- 10 kDa on SDS-PAGE.

A novel recombinant enzyme complex of the present invention can be prepared by using genetic material, i.e. recombinant DNA fragments originated from a microorganism identified as *G. oxydans* DSM 4025 or a biologically and/or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of *G. oxydans* DSM 4025. Such a novel cytochrome c oxidase complex may comprise at least one recombinant polypeptide as one of the core subunits. The recombinant polypeptide as the core subunit I of the said complex can be selected from the group consisting of polypeptide having an amino acid sequence identified by SEQ ID NO: 2 and those having amino acid sequences having 85% or higher identity with the said sequence and being capable of providing the complex with cytochrome c oxidase activity. As well, either or both of the other core subunits II (COII) and III (COIII) can be recombinant polypeptide(s). COII may be selected from the group consisting of recombinant polypeptides containing partial amino acid sequence identified by SEQ ID NO: 4 and those containing partial amino acid sequence having 85% or higher identity with the said amino acid sequence, as long as such recombinant polypeptides are capable of providing the complex with cytochrome c oxidase activity. COIII may be selected from the group consisting of recombinant polypeptides containing partial amino acid sequences identified by SEQ ID NOs: 6 and 8 and those containing partial amino acid sequences having 85% or higher identity with the respective amino acid sequences, as long as such recombinant polypeptides are capable of providing the complex with cytochrome c oxidase activity.

The term "identity" has preferably the meaning that the amino acids occurring at the respective positions are not only similar with regard to their properties but are in fact identical. In a preferred embodiment the alignment of the amino acid sequences is performed in best mode.

The present invention is also directed to the polypeptides involved in the said cytochrome c oxidase complex. The polypeptides involved in the said cytochrome c oxidase complex and the amino acid sequences described in SEQ ID NOs: 2, 4, 6 and 8 showed homologies of 50-82 % at most with the polypeptides or the corresponding partial amino acid sequences involved in other cytochrome oxidases. For example, the CO I polypeptide of the present invention (SEQ ID NO: 2) showed 77%, 81% and 79% homologies with CO I a (accession No. P08305) and CO I b (accession No. P98002) from *P. denitrificans* and CO I from *R. sphaeroides* (accession No. P33517), respectively. The partial CO II polypeptide of the present invention (SEQ ID NO: 4) showed 73% and 68% with the CO II polypeptides from *P. denitrificans* and *R. sphaeroides*, respectively. The

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partial CO III polypeptide of the present invention (SEQ ID NO: 6 ) showed 54% with the CO III polypeptide from *P. denitrificans* and another polypeptide (SEQ ID NO: 8) showed 71% and 63% with the CO III polypeptides from *P. denitrificans* and *R. sphaeroides*, respectively. These homology searches can be done by a computer  
5 programm such as "Search Homology" of Genetyx-SV/RC version 3.2.0 (Genetyx Software Development Co. Ltd., Tokyo Japan).

Thus the respective core subunits, i.e. COI, COII and COIII may be provided as recombinant polypeptides which are useful for the components of the novel cytochrome c oxidase complex of the present invention.

10 The subunit, COI of the complex may be a recombinant polypeptide which is involved in cytochrome c oxidase complex of the present invention, which polypeptide comprises an amino acid sequence identified by SEQ ID NO: 2 or an amino acid sequence having 85 % or higher identity with the said amino acid sequence and is capable of providing the complex with cytochrome c oxidase activity, as described above. A  
15 recombinant COI may also be a polypeptide capable of providing the complex of the present invention with cytochrome c oxidase activity, which is encoded by a recombinant DNA fragment comprising a DNA sequence selected from the group consisting of:  
(a) the DNA sequence identified by SEQ ID NO: 1, and  
(b) the DNA sequences which encode polypeptides having an amino acid sequence  
20 identified by SEQ ID NO: 2 or amino acid sequences having 85 % or higher identity with the said amino acid sequence.

Also the subunit, COII may be a recombinant polypeptide which is involved in cytochrome c oxidase complex of the present invention, which polypeptide contains an amino acid sequence identified by SEQ ID NO: 4, or an amino acid sequence having 85 %  
25 or higher identity with the said amino acid sequence and is capable of providing the said complex with cytochrome c oxidase activity, as described above. A recombinant COII may also be a polypeptide capable of providing the complex of the present invention with cytochrome c oxidase activity, which is encoded by a recombinant DNA fragment containing a DNA sequence selected from the group consisting of:  
30 (a) the DNA sequence identified by SEQ ID NO: 3, and  
(b) the DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 4 or amino acid sequences having 85 % or higher identity with the said amino acid sequence.

Moreover, the subunit, COIII may be a recombinant polypeptide which is involved  
35 in cytochrome c oxidase complex of the present invention, which polypeptide contains either or both of the amino acid sequences identified by SEQ ID NOS: 6 and 8, respectively

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- or amino acid sequences having 85 % or higher identity with the respective amino acid sequences of SEQ ID NOs: 6 and 8, as long as such recombinant polypeptides are capable of providing the said complex with cytochrome c oxidase activity, as described above. A recombinant COIII may also be a recombinant polypeptide capable of providing the
- 5 complex of the present invention with cytochrome c oxidase activity, which is encoded by a recombinant DNA fragment containing one or more DNA sequence(s) selected from the group consisting of:
- (a) the DNA sequence identified by SEQ ID NO: 5,
  - (b) the DNA sequence identified by SEQ ID NO: 7,
  - 10 (c) the DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 6 or amino acid sequences having 85 % or higher identity with the said amino acid sequence, and
  - (d) the DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 8 or amino acid sequences having 85 % or higher identity with
  - 15 the said amino acid sequence.

Furthermore the present invention encompasses functional derivatives of the recombinant polypeptides described above. Such functional derivatives are defined on the basis of the amino acid sequence of the present invention by addition, insertion, deletion and/or substitution of one or more amino acid residues of such sequences where

20 cytochrome c oxidase complex comprising such derivatives still have cytochrome c oxidase activity measured by an assay known in the art or specifically described herein. Such functional derivatives can be made either by chemical peptide synthesis or chemical modification of protein known in the art or by recombinant means on the basis of the DNA sequences as disclosed herein by methods known in the state of the art and disclosed,

25 e.g. by Sambrook et al. (s. a.). Amino acid exchanges in proteins and peptides which do not generally alter the activity of such molecules are known in the state of the art and are described, for example, by H. Neurath and R.L. Hill in "The Proteins" (Academic Press, New York, 1979, see especially Figure 6, page 14). The most commonly occurring exchanges are: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val,

30 Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly as well as these in reverse.

The present invention is directed to recombinant DNA fragments which encode the said recombinant polypeptides involved in the said cytochrome c oxidase complex that is

35 one of the essential components mediating electron transfer in the respiratory chain.



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The recombinant DNA fragments which are useful for preparing the respective core subunits, i.e. COI, COII and COIII by genetic engineering are provided. Such recombinant polypeptides are useful for the components to be involved in the novel cytochrome c oxidase complex of the present invention.

5 A recombinant DNA fragment for COI may be a DNA fragment which encodes a polypeptide involved in cytochrome c oxidase complex and comprises a DNA sequence selected from the group consisting of:

- (a) the DNA sequence identified by SEQ ID NO: 1, and
- (b) the DNA sequences which encode polypeptides having an amino acid sequence
- 10 identified by SEQ ID NO: 2 or amino acid sequences having 85 % or higher identity with the said amino acid sequence.

A recombinant DNA fragment for COII may be a DNA fragment which encodes a polypeptide involved in cytochrome c oxidase complex and contains a DNA sequence selected from the group consisting of:

- 15 (a) the DNA sequence identified by SEQ ID NO: 3, and
- (b) the DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 4 or amino acid sequences having 85 % or higher identity with the said amino acid sequence.

Moreover, a recombinant DNA fragment for COIII may be a DNA fragment which

20 encodes a polypeptide involved in cytochrome c oxidase complex and contains one or more DNA sequence(s) selected from the group consisting of:

- (a) the DNA sequence identified by SEQ ID NO: 5,
- (b) the DNA sequence identified by SEQ ID NO: 7,
- (c) the DNA sequences which encode polypeptides having an amino acid sequence
- 25 identified by SEQ ID NO: 6 or amino acid sequences having 85 % or higher identity with the said amino acid sequence, and
- (d) the DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 8 or amino acid sequences having 85 % or higher identity with the said amino acid sequence.

30

The recombinant DNA fragment of this invention may also include a DNA sequence which is capable of hybridizing to SEQ ID NO: 1, 3, 5, or 7 under standard stringency conditions which are described in more detail below.

"Standard conditions" for hybridization mean the condition which are generally used

35 by a person skilled in the art to detect specific hybridization signals and which are

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described, e. g. by Sambrook et al., "Molecular Cloning" second edition, Cold Spring Harbour Laboratory Press 1989, New York, or preferably so called stringent hybridization and non-stringent washing conditions or more preferably so called moderately stringent conditions or even more preferably so called stringent hybridization and stringent washing  
5 conditions a person skilled in the art is familiar with and which are described, e.g. in Sambrook et al (s.a.).

The present invention also provides an expression vector comprising one or more of the above mentioned recombinant DNA fragments, which vector is suitable for the expression in an organism, pro- or eukaryotic host cell. Such an expression vector can be  
10 constructed by inserting one or more of the above mentioned recombinant DNA fragments into a suitable vector which may carry expression control elements as it is well known in the art.

Further, a recombinant organism of the present invention may be prepared by introducing an expression vector mentioned above to an appropriate host cell. Such a  
15 recombinant organism of the invention will be useful for the genetic preparation of a recombinant cytochrome c oxidase complex of the present invention and also applicable to a process for producing 2KGA from L-sorbose or D-sorbitol in an appropriate culture medium. Host cells for the recombinant organism of the present invention may be of eukaryotic origin, preferably a mammalian or plant cell, or may be of prokaryotic origin.  
20 These host cells may in particular be obtained from bacteria, preferably *G. oxydans* DSM No. 4025 and biologically and/or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of *G. oxydans* DSM 4025. As well, host cells may be selected from the group consisting of bacteria, such as *Escherichia coli*, *Pseudomonas putida*, *Acetobacter xylinum*, *Acetobacter pasteurianus*, *Acetobacter aceti*,  
25 *Acetobacter hansenii*, and *G. oxydans*.

In addition it is an object of the present invention to provide a process for producing cytochrome c oxidase, which comprises cultivating a recombinant host cell as defined above in an appropriate culture medium and recovering the cytochrome c oxidase from the culture.

30

Cytochrome c oxidase complex of the present invention is also applicable for improving 2KGA production from L-sorbose or D-sorbitol and furthermore the production of aldehydes, carboxylic acids, and ketones from corresponding substrates in the presence of alcohol and aldehyde dehydrogenase in vivo and in vitro.

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The compound 2KGA is an important intermediate for the production of L-ascorbic acid into which it can be converted according to the well-known Reichstein method. The production of 2KGA from L-sorbose or from D-sorbitol by fermentation is known [T. Hoshino et al., European Patent Application 88116156 A2]. Gluconobacter strains are known to produce 2KGA via the reaction catalyzed by sorbose and sorbosone dehydrogenases as disclosed in Agric. Biol. Chem., 54(5), 1211-1218, 1990 [T. Hoshino et al.] and in European Patent Publication No. 606621 A2 [T. Hoshino et al.]. The genes of primary dehydrogenases responsible for 2KGA formation from L-sorbose or D-sorbitol have been obtained [T. Hoshino et al., European Patent Publication 832974 A2]. Furthermore, cytochrome c functioning as an electron acceptor from the primary dehydrogenases and its gene has also been isolated [T. Hoshino et al., European Patent Application No. 97105583.5]. These dehydrogenases and cytochrome c have been used to produce 2KGA in vitro. The genes have been used to construct recombinant organisms producing 2KGA from L-sorbose and D-sorbitol; e.g. Pseudomonas putida carrying the genes of alcohol/aldehyde dehydrogenase (AADH) together with cytochrome c can produce 2KGA from L-sorbose.

Therefore the use of cytochrome c oxidase of the present invention for the production of 2KGA is also an object of the present invention.

The terminal oxidase activity of the present cytochrome c oxidase complex was spectrophotometrically measured using TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride) as an artificial substrate (electron donor). The reaction mixture e.g., consists of 2.5 mM TMPD, 0.05% Tween-20 and 0.1 M sodium 3[N-morpholino]propanesulfonic acid (Na-MOPS) (pH 6.5). The TMPD oxidase activity can be measured by increasing of absorption at 520 nm, and molar coefficient of TMPD is taken as 6.1 /mM/cm. One unit of enzyme activity is defined as 1 mmole oxidation of TMPD per one minute at room temperature.

Spectrophotometric identification and quantification of a-type heme were carried out by detection of characteristic positive peak around 605 nm by reduced minus oxidized difference spectrum. Reduction of each sample was done by addition of tiny amount of sodium dithionite, oxidation by ammonium persulfate. Molar coefficient of a-type heme peak (605 nm - 630 nm) was taken as 11.7 /mM/cm.

Before describing the present invention in more detail the physico-chemical properties of purified cytochrome c oxidase consisting of subunits, COI and COII, as obtainable from G. oxydans DSM No. 4025 are given below.

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Absorption profile of the cytochrome c oxidase complex in reduced minus oxidized difference spectra is shown in Fig. 1.

(2) Molecular weight

- 5 SDS-PAGE analysis showed apparent molecular masses of about 43 +/- 10 and 36 +/- 10 kDa for cytochrome c oxidase CO I and CO II subunits, respectively, as shown in Fig. 2.

(3) Amino acid sequences of the CO I and CO II

- 10 The cytochrome c oxidase complex purified is dissociated into CO I and II subunits by a preparative-disc-SDS-PAGE (NA-1800, Nippon Eido Co.,). Both N-terminal amino residues were blocked by unidentified modification. Then, partially digested peptide fragments (15 - 45 kDa MW.) were derived by lysyl-endopeptidase treatment, isolated by band extraction from 15% SDS-PAGE sheet, washed in Centricon-10(Amicon)  
15 with 15% methanol and 0.1% SDS, and applied to the sequencer.  
"KDIGLLYLVAAGVVGF" (SEQ ID NO: 11) and "KASQFTHNTPLEIVWTIVPV" (SEQ ID NO: 14) sequences were obtained for CO I and COII, respectively.

- A preferred strain used for isolating polypeptides and genes of cytochrome c oxidase of the present invention is *G. oxydans* strain that has been deposited at the Deutsche  
20 Sammlung von Mikroorganismen in Göttingen (Germany) under DSM No. 4025 on March 17, 1987. Moreover, a subculture of the strain has also been deposited in the Agency of Industrial Science and Technology, Fermentation Research Institute, Japan, under the stipulations of the Budapest Treaty under the deposit No.: *Gluconobacter oxydans* DSM No. 4025 FERM BP-3812 (date of deposit: March 30, 1992). Furthermore,  
25 European Patent Publication 0278 477 discloses the characteristics of this strain. Functional equivalents, subcultures, mutants and variants of said microorganism can be used in the present invention. Biologically or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of the strain DSM 4025 can be also used as the source of the polypeptides and genes of the said cytochrome c oxidase.

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The cytochrome c oxidase provided by the present invention can be prepared by cultivating an appropriate organism, disrupting the cells and isolating and purifying it from cell free extract of disrupted cells, preferably from the soluble fraction of the organism.

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The organisms may be cultured in an aqueous medium supplemented with appropriate nutrients under aerobic conditions. The cultivation may be conducted at pH between about 4.0 and 9.0, preferably between about 6.0 and 8.0. While the cultivation period varies depending upon pH, temperature and nutrient medium used, usually 2 to 6  
5 days will bring about favorable results. A preferred temperature range for carrying out the cultivation is from about 13° to 36°C, preferably from about 18° to 33°C.

It is usually required that the culture medium contains such nutrients as assimilable carbon sources, digestible nitrogen sources and inorganic substances, vitamins, trace elements and the other growth promoting factors. As assimilable carbon sources, glycerol,  
10 D-glucose, D-mannitol, D-fructose, D-arabitol, L-sorbose, D-sorbitol and the like can be used.

Various organic or inorganic substances may also be used as nitrogen sources, such as yeast extract, meat extract, peptone, casein, corn steep liquor, urea, amino acids, nitrates, ammonium salts and the like. As inorganic substances, magnesium sulfate,  
15 potassium phosphate, ferrous and ferric chlorides, calcium carbonate and the like may be used.

In the following, embodiments for the isolation and purification of cytochrome c oxidase from the organisms after the cultivation and for its cloning of the gene/DNA sequence are described.  
20

- (1) Cells are harvested from the fermentation broth by centrifugation or filtration.
- (2) The cells are suspended in the buffer solution and disrupted by means of a homogenizer, sonicator or treatment with lysozyme and the like to give a disrupted  
25 solution of cells.
- (3) Cytochrome c oxidase is isolated and purified from a cell free extract of disrupted cells, preferably from the soluble fraction of the organisms by usual protein purification methods such as ammonium sulfate precipitation, dialysis, ion exchange  
30 chromatographies, gel filtration chromatographies, and affinity chromatographies.

The cytochrome c oxidase provided by the present invention is useful as a terminal oxidase oxidizing cytochrome c, an electron acceptor from an enzyme belonging to dehydrogenase for the production of aldehydes, carboxylic acids and ketones from

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alcohols and aldehydes, especially for the production of 2KGA from L-sorbose or D-sorbitol via L-sorbose.

Briefly, the cytochrome c oxidase genes, the DNA sequences, the recombinant expression vector and the recombinant organism, also called transformed host cell, utilized in the present invention can be obtained by the following steps:

- (1) Isolating chromosomal DNA from the organisms which can provide cytochrome c oxidase of the present invention and constructing the gene library with the chromosomal DNA in *Escherichia coli*.
- (2) Cloning cytochrome c oxidase genes from a chromosomal DNA by colony-, plaque-, or Southern-hybridization, PCR (polymerase chain reaction) cloning, Western-blot analysis and the like.
- (3) Determining the nucleotide sequences of the cytochrome c oxidase genes obtained as above by usual methods to select recombinant DNA fragments containing said cytochrome c oxidase genes and constructing the expression vector on which cytochrome c oxidase genes can express efficiently.
- (4) Constructing recombinant organisms carrying cytochrome c oxidase gene by transformation, transduction, transconjugation and electroporation.

The materials and the techniques used in the above aspect of the present invention are exemplified in details as follows:

A total chromosomal DNA can be purified by a procedure well known in the art. The genes encoding cytochrome c oxidase are cloned in either plasmid or phage vectors from a total chromosomal DNA by the following methods:

- (i) by determining the partial amino acid sequences from the purified cytochrome c oxidase subunits by isolating the whole protein or peptide fragments obtained by peptidase-treatment from the gel after SDS-polyacrylamide gel electrophoresis and applying them to protein sequencer such as Applied Biosystems automatic gas-phase sequencer 470A (Perkin Elmer Corp., Norwalk, Conn., USA), synthesizing oligonucleotide probes with DNA synthesizer such as Applied Biosystems automatic DNA sequencer 381A (Perkin Elmer), according to the amino acid sequences obtained as above, isolating clones carrying the objective genes from a gene library of the strain carrying the objective genes with the oligonucleotide probes through Southern-, colony- or plaque-hybridization; (ii) by selecting clones expressing cytochrome c oxidase subunits from the gene library by

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immunological methods with antibodies against the subunits of cytochrome c oxidase; or (iii) by amplifying the DNAs from the total chromosomal DNA by PCR method with sets of two oligonucleotides synthesized according to the amino acid sequences determined as above and isolating clones carrying the whole genes of cytochrome c oxidase subunits  
5 from the gene library constructed in *E. coli* by Southern-, colony-, or plaque-hybridization with the PCR product obtained above as the probe. Above mentioned antibodies reacting against the subunits of cytochrome c oxidase can be prepared with the purified proteins of cytochrome c oxidase subunits or their peptide fragments by such method described in *Methods in Enzymology*, vol. 73, p 46, 1981.

10 The nucleotide sequences of the cytochrome c oxidase genes can be determined by a well-known method such as dideoxy chain termination method with M13 phage (Sanger F. et al., *Proc. Natl. Acad. Sci. USA*, 74:5463-5467, 1977).

To express the genes of cytochrome c oxidase complex subunits, various promoters can be used; for example, the original promoter existing upstream of the said genes for  
15 cytochrome c oxidase subunits, promoters of antibiotic resistance genes such as kanamycin resistant gene of Tn5 (Berg, D. E., and C. M. Berg. 1983. *Bio/Technology* 1: 417-435), ampicillin resistant gene of pBR322, and  $\beta$ -galactosidase of *E. coli* (*lac*), *trp*-, *tac*-, *trc*-promoter, promoters of lambda phage and any promoters which can be functional in the hosts consisting of organism including bacteria such as *E. coli*, *P. putida*,  
20 *A. xylinum*, *A. pasteurianus*, *A. aceti*, *A. hansenii*, and *G. oxydans*, especially *G. oxydans* DSM No. 4025, mammalian cells and plant cells.

For the object above other regulatory elements such as a Shine-Dalgarno (SD) sequence (for example, AGGAGG etc. including natural and synthetic sequences operable in the host cell) and a transcriptional terminator (inverted repeat structure including any  
25 natural and synthetic sequence operable in the host cell) which are operable in the host cell into which the coding sequence will be introduced and used with the above described promoter.

A wide variety of host/cloning vector combinations may be employed in cloning the double-stranded DNA. Cloning vector is generally a plasmid or phage which contains an  
30 replication origin, regulatory elements, a cloning site including a multi-cloning site and selection markers such as antibiotic resistance genes including resistance genes for ampicillin, tetracycline, kanamycin, streptomycin, gentamicin, spectinomycin etc.

Preferred vectors for the expression of the object gene in *E. coli* are selected from any vectors usually used in *E. coli*, such as pBR322 or its derivatives including pUC18 and  
35 pBluescript II, pACYC177 and pACYC184 [*J. Bacteriol.*, 134:1141-1156, 1978] and their

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derivatives, and a vector derived from a broad host range plasmid such as RK2 and RSF1010. A preferred vector for the expression of the object gene in *Gluconobacter* including *G. oxydans* DSM No. 4025 and *P. putida* is selected from any vectors which can replicate in *Gluconobacter* and/or *P. putida*, as well as in a preferred cloning organism  
5 such as *E. coli*. The preferred vector is a broad-host-range vector such as a cosmid vector like pVK102 and its derivatives and RSF1010 and its derivatives, and a vector containing a replication origin functional in *Gluconobacter* and another origin functional in *E. coli*. Copy number and stability of the vector should be carefully considered for stable and efficient expression of the cloned gene and also for efficient cultivation of the host cell  
10 carrying the cloned gene. DNA sequences containing transposable elements such as Tn5 can be also used as a vector to introduce the object gene into the preferred host, especially on a chromosome. DNA sequences containing any DNAs isolated from the preferred host together with the object gene are also useful to introduce the desired DNA sequence into the preferred host, especially on a chromosome. Such DNA sequences can be transferred  
15 to the preferred host by transformation, transduction, transconjugation or electroporation.

Useful hosts are of pro- or eukaryotic origin and may include organisms, mammalian cells, and plant cells. As a preferable organism, there may be mentioned bacteria such as *E. coli*, *P. putida*, *A. xylinum*, *A. pasteurianus*, *A. aceti*, *A. hansenii*, *G.*  
20 *oxydans*, and any Gram-negative bacteria which are capable of producing recombinant cytochrome c oxidase. Functional equivalents, subcultures, mutants and variants of said organism can be also used in the present invention. A preferred strain is *E. coli* K12 and its derivatives, *P. putida* or *G. oxydans* DSM No. 4025 and biologically or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of strain  
25 DSM 4025.

The DNA sequence encoding cytochrome c oxidase of the present invention is ligated into a suitable vector containing a regulatory region such as a promoter and a ribosomal binding site and transcriptional terminator operable in the host cell described above by well-known methods in the art to produce an expression vector.

30 To construct a host cell carrying an expression vector, various DNA transfer methods including transformation, transduction, conjugal mating (Chapters 14 and 15, Methods for general and molecular bacteriology, Philipp Gerhardt et al. ed., American Society for Microbiology, 1994), and electroporation can be used. The method for constructing a transformed host cell may be selected from the methods well-known in the  
35 field of molecular biology. Usual transformation system can be used for *E. coli*, *Pseudomonas* and *Acetobacter*. Transduction system can also be used for *E. coli*. Conjugal mating system can be widely used in Gram-positive and Gram-negative bacteria



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including *E. coli*, *P. putida* and *G. oxydans*. A preferred conjugal mating method was basically disclosed by the inventor in PCT Publication No. WO89/06688. The conjugation can occur in liquid medium or on a solid surface. The preferred recipient for cytochrome c oxidase production is selected from *E. coli*, *P. putida* and *G. oxydans*. The preferred recipient for 2KGA production is selected from *E. coli*, *P. putida* and *G. oxydans* which can produce active AADHs and cytochrome c with a suitable recombinant expression vector. The preferred recipient for 2KGA production is *G. oxydans* DSM No. 4025. To the recipient for conjugal mating, a selective marker is usually added; for example, resistance against nalidixic acid or rifampicin is usually selected.

- 10        The Examples which follow are provided to further illustrate the invention and are not intended to limit the invention in any way.

Example 1:

Identification and purification of cytochrome c oxidase of *G. oxydans* DSM4025

- 15        Terminal oxidase activity was spectrophotometrically measured using TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride) as an artificial substrate (electron donor). The reaction mixture consisted of 2.5 mM TMPD, 0.05% Tween-20 and 0.1 M sodium 3[N-morpholino]propanesulfonic acid (Na-MOPS) (pH 6.5). TMPD oxidase activity was measured by increasing of absorption at 520 nm, and mole coefficient of TMPD was taken as 6.1 /mM/cm. One unit of enzyme activity was defined as 1 mmole oxidation of TMPD per one minute at room temperature. Spectrophotometric identification and quantification of a-type heme were carried out by analysing a reduced minus oxidized difference spectrum to detect the characteristic positive peak around 605 nm. Each sample was reduced with sodium dithionite and oxidized with ammonium persulfate. Mole coefficient of a-type heme peak (605 nm - 630 nm) was taken as 11.7 /mM/cm.
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- 25

- G. oxydans* DSM4025 was aerobically cultivated with 5 liter of FYC medium, which consisted of 10% L-sorbose (separately sterilized), 0.05% glycerol, 1.6% urea (separately sterilized), 0.25% MgSO<sub>4</sub>·7H<sub>2</sub>O, 6.25% baker's yeast cells, 1.5% CaCO<sub>3</sub> (production grade, nacalai tesque, Kyoto, Japan), and 3.0% corn steep liquor, pH 7.5 (before sterilization) at 30°C for 27 hours. After the cultivation, solid materials such as CaCO<sub>3</sub> and yeast cells were precipitated by low speed centrifugation (1,000 rpm for 5 minutes) and removed. *G. oxydans* DSM4025 cells remained in the culture supernatant were collected by centrifugation at 8,000 rpm for 20 minutes and once washed with 25 mM sodium N-[2-hydroxyethyl] piperazine-N'-[4-butanesulfonic acid] (Na-HEPES) (pH 7.5) containing 0.25 M NaCl, and 2 mM MgCl<sub>2</sub>.
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The resulting cells (about 35 g wet weight) were suspended in about 200 ml of 25 mM Na-HEPES (pH 7.5) containing 0.5 mM ethylenediamine tetraacetic acid (EDTA), 0.5 mM ethylene glycol-bis-b-aminoethyl ether (EGTA) 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml pepstatin A, 1 mg/ml leupeptin, 10 mg/ml DNase I and 10 mg/ml RNase A. The cell suspension was treated with French press homogenizer at 1500 kg/cm<sup>2</sup> twice. The resulting suspension was centrifuged at 10,000 rpm for 10 minutes to remove cell debris, and the supernatant was collected as cell-free extract (424.0 mg proteins). The cell-free extract was subjected to ultra-centrifugation operated at 55000 rpm for 1 hour to recover the precipitate as a crude membrane fraction. The crude membrane fraction was resuspended in 50 ml of 25 mM Na-HEPES (pH 7.5) containing 1.2% Tween 20, 0.25 M NaCl, 2 mM MgCl<sub>2</sub>, 0.5 mM PMSF, 1 mg/ml pepstatin A 1 mg/ml leupeptin and incubated for 1 hour for washing the membrane. The fraction was again subjected to ultra-centrifugation operated at 55,000 rpm for 1 hour to recover the precipitate as a washed membrane fraction. The washed membrane fraction was incubated with 50 ml of 25 mM Na-HEPES (pH 7.5) containing 1.5% sucrose monolaurate (DOJIN Laboratories, Kumamoto, Japan), 2 mM EDTA and 5% glycerol for 1 hour to solubilize the membrane-bound proteins and the resulting suspension was subjected to ultra-centrifugation operated at 55,000 rpm for 1 hour to obtain a supernatant (50 ml) as solubilized membrane fraction. Reduced minus oxidized difference spectrum of the solubilized membrane fraction showed characteristic positive peak around 605 nm; the peak corresponds to 0.41 nmoles of a-type heme/mg of crude proteins as content. Then, membrane-bound proteins in the solubilized membrane fraction were load on a DEAE-Toyopearl 650M (TOSOH, Tokyo, Japan) column (ID 2.2 x 5 cm) which had been equilibrated with 25 mM Na-HEPES containing 0.5% sucrose monolaurate and 5% glycerol. Fractionation was carried out by a liner gradient of 0 - 0.35 M NaCl in the same buffer. Fractions showing a-type heme spectra (positive peak around 605 nm on reduced minus oxidized difference spectrum) and TMPD oxidase activity were eluted around 0.28 M concentration of NaCl. These fractions were collected (64 ml), dialyzed against 45 mM potassium phosphate buffer [KPB] (pH 7.6) containing 45 mM NaCl, 5% glycerol and 0.5% sucrose monolaurate, and the enzyme solution was loaded on hydroxylapatite (TONEN Co., Tokyo, Japan) column (ID 1.5 x 6 cm) which had been equilibrated with the same buffer. The column was washed with the same buffer and then with 500 mM KPB (pH 7.6) containing 500 mM NaCl, 5% glycerol and 0.5% sucrose monolaurate, and the active fractions were eluted with 900 mM KPB (pH 7.6) containing 900 mM NaCl, 5% glycerol and 0.5% sucrose monolaurate and collected. The fraction (8.6 mg protein) from the hydroxylapatite column was dialyzed against 25 mM Na-HEPES (pH 7.5) containing 0.5% sucrose monolaurate and 5% glycerol, concentrated by ultrafiltration using YM-30 membrane (Amicon Inc., MA, USA) and stored at -30°C as a purified protein.

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The purified protein was subjected to native-polyacrylamide gel electrophoresis (Native-PAGE) analysis in the presence of 0.5% sucrose monolaurate; the protein showed a visible band with greenish color (without protein staining) which corresponded to a single protein band (with protein staining). The purified protein had 2.6 units/mg of  
5 TMPD oxidase activity and showed typical absorption spectra pattern of aa3-type cytochrome c oxidase (Fig. 1). Content of a-type heme was estimated to be 19.2 nmoles/mg of the purified protein. Purification fold on the content of a-type heme from the washed membrane was about 100-folds with nearly 90% recovery. These results indicated that the purified protein was a major component with TMPD-oxidase activity in  
10 *G. oxydans* DSM4025, which can function as a terminal oxidase on respiratory system. In SDS-PAGE analysis, the purified protein was disassociated into two protein components: one showed a broad band with an apparent molecular weight of about 43,000 (named as CO I) and the other showed a sharp band with apparent molecular weight of about 36,000 (named as CO II). (Fig. 2)

15

#### Example 2:

Amino acid sequences of cytochrome c oxidase of *G. oxydans* DSM4025 and the homologies with the other cytochrome c oxidase complexes.

Two components (CO I and CO II) of the purified cytochrome c oxidase of *G.*  
20 *oxydans* DSM4025 were disassociated by a preparative SDS-PAGE. From both components, native N-terminal amino acid sequences were not obtained. To obtain the internal amino acid sequence, each component was digested by lysyl-endopeptidase and the resulting fragments were isolated by preparative SDS-PAGE and subjected to amino acid sequencing with an amino acid sequencer (Applied Biosystems model 470A, The  
25 Perkin Elmer Corp., Conn., USA). As the results, partial amino acid sequences were obtained; KDIGLLYLVAAGVVGF [SEQ ID NO: 11], was obtained from the CO I fragment (slightly lower molecular weight from the original) and KASQFTHNTPLEIVWTIVPV [SEQ ID NO:14], was from the CO II fragment (about 10000 lower molecular weight from the original). The partial amino acid sequences of the  
30 CO I and CO II subunits were compared with the total amino acid sequence of cytochrome c oxidase complexes of *P. denitrificans* and *R. sphaeroides* and bovine mitochondria by sequence-alignments (Figs. 3 to 4) because the CO I and CO II were similar to those of cytochrome c oxidase of *P. denitrificans* (B. Ludwig and G. Schatz, Proc. Natl. Acad. Sci. USA, 77, 196-200, 1980) in the SDS-PAGE and spectrophotometric  
35 characters. Total homology in the amino acid sequences among three cytochrome c oxidase complexes had been previously reported (C. Jianli et al., J. Biol. Chem., 267, 24273-24278, 1992). As shown in Figs. 3 and 4, the amino acid sequences of *G. oxydans*

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DSM4025 cytochrome c oxidase CO I and CO II were partially assigned to the homology alignment of the others. Especially, significant homology was observed with two bacterial sequences (*P. denitrificans* and *R. sphaeroides*).

5 Example 3:

Cloning of cytochrome c oxidase genes of *G. oxydans* DSM4025

(1) Amplification of partial cytochrome c oxidase gene(s) by the PCR method.

According to the total amino acid sequence alignments of *P. denitrificans*, *R.*  
10 *sphaeroides* and bovine mitochondria together with the amino acid sequences of the purified CO I and CO II polypeptides (SEQ ID NOs: 11 and 14), the following amino acid sequences were selected for PCR primers to amplify partial DNA sequences of CO I and CO II genes: SEQ ID NO: 9 and SEQ ID NO:10 for the CO I gene; and SEQ ID NO: 15 and SEQ ID NO:16 for the CO II gene. The third component (CO III), which was reported to  
15 be included in cytochrome c oxidase complex, did not exist in the preparation purified from *G. oxydans* DSM4025; the absence seemed to be due to disassociation during the purification. To confirm and amplify a partial DNA sequence encoding the assumed CO III gene of *G. oxydans* DSM4025, if it exists, two amino acid sequences, which were conserved in the polypeptides encoded by three CO III genes of *P. denitrificans*, *R.*  
20 *sphaeroides* and bovine mitochondria, were selected (Fig. 5.): SEQ ID NO: 17 and SEQ ID NO:18 for CO III gene. Each pair of primers was designed for CO I, CO II or CO III (Fig. 6). The PCR reaction was carried out by using the GeneAmp™ DNA Amplification Reagent Kit (Takara Shuzo, Kyoto, Japan) with the Parkin-Elmer Cetus Instruments Thermal Cycler according to the recommendations of the supplier. The reaction consisted  
25 of 30 cycles of 1) denaturation step at 94°C for 1 minute; 2) annealing step at 42 or 50°C for 2 minutes; and 3) synthesis step at 72°C for 3 minutes. The reaction mixture (100 µl) contained 200 mM of dNTPs, 2.9 mM (for 32 degeneracy) or 5.8 mM (for 64 degeneracy) of each primer, 2.2 ng of chromosomal DNA of *G. oxydans* DSM4025, and 2.5 units of Taq polymerase in the buffer supplied. PCR product was detected by agarose gel  
30 electrophoresis [AGE] with ethidium bromide staining. As a result, DNA fragments with expected length (about 180 bp for CO I, about 180 bp for CO I, about 300 bp for CO III) were amplified.

(2) Cloning and nucleotide sequencing of the DNA fragments amplified by PCR

35 The PCR-amplified DNA fragments were purified from an agarose gel and directly cloned into pCRTMII vector (Invitrogen Corporation, USA), and the DNA sequences

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were determined according to the supplier's instruction. Amino acid sequences deduced from the nucleotide sequences of the PCR products showed considerable homologies with the sequences of target positions in the alignments (Figs. 3 to 5). The PCR products encoding the partial amino acid sequences of CO I, CO II and CO III were labeled with <sup>32</sup>P to obtain probes Pco1, Pco2, and Pco3, respectively. The probes were used for Southern- or colony-hybridization to detect the complete CO I, CO II, and CO III genes.

(3) Southern-blot analysis of the *G. oxydans* DSM4025 chromosomal DNA using the PCR products as probes

10 The chromosomal DNA of *G. oxydans* DSM4025 digested with various restriction endonucleases was subjected to Southern hybridization using the probes. The probe Pco1 hybridized to a Pst I fragment (8.0 kb), and the probes Pco2 and Pco3 hybridized to an EcoRI fragment (9.3 kb).

15 (4) Cloning of complete cytochrome c oxidase genes in the 8.0 kb PstI fragment (CO I) and the 9.3 kb EcoRI fragment (CO II and CO III)

The chromosomal DNA of *G. oxydans* DSM 4025 was completely digested with PstI or EcoRI and the resulting fragments were subjected to agarose gel electrophoresis. EcoRI-digests around 9.3 kb (7-12 kb) in size and PstI-digests around 8 kb (6-10 kb) were cut out and eluted from the gel. The recovered DNA fragments were ligated with PstI- or EcoRI-digested pUC19 vector to transform *E. coli* JM109. About 1,000 transformants were obtained as PstI- or EcoRI-library. Colony hybridization was performed with the probe Pco1 for the PstI library and with the primers Pco2 and Pco3 for the EcoRI library. From each library, several positive colonies were obtained. Plasmid DNAs were extracted from the colonies and digested with PstI or EcoRI; 8.0 kb PstI fragment showed a strong signal with the probe Pco1, and 9.3 kb EcoRI fragment showed a strong signal with both of the probes Pco2 and Pco3. The plasmid containing 8.0 kb PstI fragment were designated as pUCO01 and the plasmid containing 9.3 kb EcoRI fragment as pUCO23.

30 (5) Physical map of the 8.0 kb PstI and 9.3 kb EcoRI fragments

Physical maps of the 8.0 kb PstI and 9.3 kb EcoRI fragments were constructed by the Southern hybridization analysis of the fragments digested with various restriction endonucleases with the probes Pco1, Pco2 and Pco3. Direction and distance of CO II and CO III genes encoded on the 9.3 kb EcoRI fragment was determined by the PCR method with primers derived from the partial nucleotide sequences (Fig. 7).

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(6) Nucleotide sequencing of the complete CO I gene

The nucleotide sequence of the COI genes on pUCO01 was determined by the dideoxy chain termination method. A 2.9 kb fragment upstream from HindIII site as shown in Fig. 7 was sequenced and one open reading frame (CDS of 1,674 bp existing in the sequence shown in SEQ ID NO: 1) was found in the fragment. This ORF encodes a protein of 558 amino acids (sequence list SEQ ID NO: 2), containing the stretch consistent with the amino acid sequence (SEQ ID NO: 11) of the peptide fragment derived from the purified CO I and the amino acid sequence (SEQ ID NO: 13) deduced from the DNA sequence of the about-180 bp PCR product (SEQ ID NO: 12) for CO I [see 3-(1)] The CO I amino acid sequence of *G. oxydans* DSM4025 showed 78.7, 76.0 and 53.3% homologies with those of *R. sphaeroides*, *P. denitrificans* and bovine mitochondria, respectively. (Fig. 8)

(7) Construction of an expression plasmid encoding all of CO I, CO II, and CO III genes

CO I gene was isolated from the 8.0 kb PstI fragment on pUCO01 by complete HindIII- and partial-EcoRI digestions as a 3.5 kb fragment (Fig. 7). According to the physical map of the 9.3 kb EcoRI fragment on pUCO23, CO II and CO III genes were isolated by complete-KpnI and partial-PstI digestions to make a 6.0 kb fragment in tandem form (Fig. 7). Each fragment was once subcloned into a pBluescriptII SK+ vector to obtain plasmids pBCO01 with 3.5 kb fragment containing CO I gene and pBCO23 with 6.0 kb fragment containing CO II and CO III genes.

As shown in Fig. 9, the 3.5 kb fragment containing CO I gene and the 6.0 kb fragment containing CO II and CO III genes were co-integrated in one expression vector for functional expression of genes of the cytochrome c oxidase complex (CO I, CO II, and CO III). At first, the 6.0 kb XbaI - KpnI fragment containing CO II and CO III genes from pBCO23 was inserted in the EcoRI site of plasmid vector, pVK101 by the blunt end ligation. Then, the 3.5 kb XbaI - HindIII fragment containing CO I gene from pBCO01 was inserted in the BglII site of pVK101 with the 6.0 kb XbaI - KpnI fragment. The resulting plasmid vector was designated as pVKcoxes.

Example 4:

Overexpression of cytochrome c oxidase genes in *G. oxydans* DSM 4025 derivative

The plasmid carrying the cytochrome c oxidase genes in pVK101, pVKcoxes, was introduced into a rifampicin resistant derivative of *G. oxydans* DSM4025, GOS2RPM [a

- 25 -

single colony isolate from GOS2R; T. Hoshino et al., European Patent Publication 832974 A2] by the tri-parental conjugal mating method. Cells of GOS2RPM were cultivated at 30°C in 10 ml of T medium consisted of 3% Trypticase Soy Broth (Bacton Dickinson, Cockeysville, Md., USA) and 0.3% yeast extract (Difco Laboratories, Detroit, Mich.) with 100 mg/ml of rifampicin (TR medium). A donor strain, *E. coli* HB carrying pVKcoxes (Tcr, Kmr) or pVK102 (Tcr, Kmr) and a helper strain, *E. coli* HB101 carrying pRK2013 (Kmr) were grown in Luria Bertani medium containing appropriate antibiotics overnight at 37°C. These overnight cultures (10 ml of GOS2RPM culture and 2 ml of *E. coli* culture) were independently centrifuged and cell pellets were independently suspended in 2 ml of T medium. One hundred ml of the cell suspensions were mixed and 50 ml of the mixed cell suspension were spotted onto a nitrocellulose filter placed on the surface of NS2 agar medium consisted of 5.0% D-mannitol, 0.25% MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.75% corn steep liquor, 5% baker's yeast (Oriental Yeast Co., Tokyo, Japan), 0.5% CaCO<sub>3</sub>, 0.5% urea (separately sterilized), and 2.0% agar, pH 7.0 (before sterilization). The plate was incubated at 27°C overnight. The resulting cells were spread onto T agar medium containing 100 mg/ml rifampicin and 3 mg/ml tetracycline (TRT agar plate). The transconjugants thus obtained were purified by streaking on TRT agar plate to remove cells of *E. coli* and plasmid-free GOS2RPM.

The resulting transconjugants, GOS2RPM (pVKcoxes) and GOS2R (pVK102) were cultivated, and cells of both transconjugants were prepared according to the method described in Example 1. Cytochrome c oxidase level in GOS2RPM (pVKcoxes) was determined by the following experiments in comparison with that of GOS2RPM (pVK102). From both strains, solubilized membrane fraction was prepared by the method described in Example 1. First, a-type heme contents were determined to be 0.031 and 0.022 nmoles/mg of cell proteins for GOS2RPM (pVKcoxes) and GOS2R (pVK102), respectively, by the reduced minus oxidized difference spectrum method (Example 1). Second, specific oxidation rate of cytochrome c (purchased from Sigma, horse heart type VI) was measured. Reduced cytochrome c was prepared by sodium dithionite as a reducer, and excess reducer was removed by twice treatment of PD-10 column (Pharmacia). Reaction mixture consisted of 33 mM reduced cytochrome c, 25 mM Na-HEPES (pH 7.2), 2% sucrose monolaurate and 0.5 mM EDTA. Oxidation rate of the reduced cytochrome c was measured by decrease of absorbance at 550 nm, and mole coefficient was taken as 21.1 /mM/cm. Specific oxidation rates on the reduced cytochrome c were determined to be 1.58 and 2.00 nmoles/mg cell proteins/min for GOS2RPM (pVK102) and GOS2R (pVKcoxes), respectively. Third, contents of CO I and CO II components were compared by the Western-blot analysis with the antibody against the component CO I or CO II. Stronger band intensifies (60% increase for CO I and 41% increase for CO II by using CCD camera) were observed on GOS2RPM (pVKcoxes). These results suggested that

- 26 -

introduction of pVKcoxs resulted in functional amplification of the cytochrome c oxidase complex level in the 2KGA producing *G. oxydans* DSM 4025 derivative.



- 27 -

## SEQUENCE LISTING

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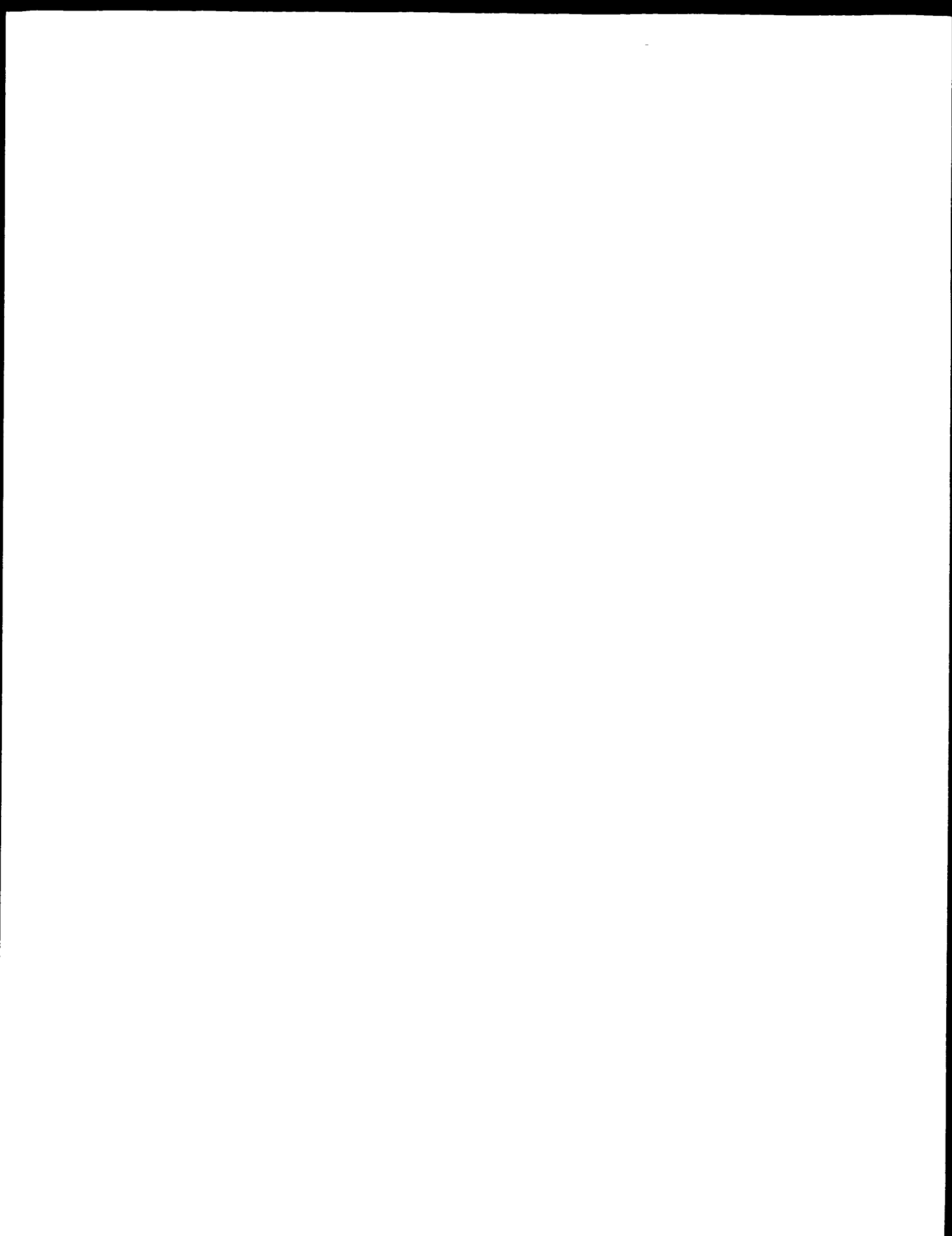
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- 42 -

EPO - Munich  
59

17. Nov. 1999

Claims

1. Cytochrome c oxidase complex having cytochrome c oxidase activity obtainable or obtained from biological or genetic material originated from a microorganism identified  
5 as *Gluconobacter oxydans* DSM 4025 or biologically and/or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of *Gluconobacter oxydans* DSM 4025.

2. Cytochrome c oxidase complex as set forth in claim 1, which shows the following physicochemical properties of:

- 10 (i) showing the presence of at least two core subunits of I (COI) and II (COII) wherein; apparent molecular mass of COI is about 43 +/- 10 kDa by SDS-PAGE analysis; and apparent molecular mass of COII is about 36 +/- 10 kDa by SDS-PAGE analysis and  
(ii) absorption spectrum showing aa3-type cytochrome c oxidase being;  
605 +/- 1 nm peak in reduced minus oxidized difference spectrum.

15 3. Cytochrome c oxidase complex as set forth in claim 1 or 2, which is substantially homogeneous isolate derived from a culture of a microorganism identified as *Gluconobacter oxydans* DSM 4025 or biologically and/or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of *Gluconobacter oxydans* DSM 4025.

20 4. Cytochrome c oxidase complex as set forth in any one of claims 1 - 3, which is a recombinant enzyme.

5. Cytochrome c oxidase complex as set forth in claim 4, which comprises a recombinant polypeptide selected from the group consisting of polypeptides having an amino acid sequence identified by SEQ ID NO: 2 and those having amino acid sequences  
25 having 85% or higher identity with the said sequence and being capable of providing the complex with cytochrome c oxidase activity.

6. Cytochrome c oxidase complex as set forth in claim 4 or 5, which contains at least one recombinant polypeptide(s) selected from the group consisting of those containing amino acid sequences identified by SEQ ID NOs: 4, 6 and/or 8 and those containing amino  
30 acid sequences having 85% or higher identity with any one of the said amino acid sequences, and are capable of providing the complex with cytochrome c oxidase activity.

7. A recombinant polypeptide which is involved in cytochrome c oxidase complex described in any one of claims 1 - 6, which polypeptide comprises an amino acid sequence

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identified by SEQ ID NO: 2 or an amino acid sequence having 85 % or higher identity with the said amino acid sequence and is capable of providing the complex with cytochrome c oxidase activity.

8. A recombinant polypeptide as set forth in claim 7 capable of providing the  
5 complex described in any one of claims 1 - 6 with cytochrome c oxidase activity, which is encoded by a recombinant DNA fragment comprising a DNA sequence selected from the group consisting of:

- (a) the DNA sequence identified by SEQ ID NO: 1, and
- (b) the DNA sequences which encode polypeptides having an amino acid sequence  
10 identified by SEQ ID NO: 2 or amino acid sequences having 85 % or higher identity with the said amino acid sequence.

9. A recombinant polypeptide which is involved in cytochrome c oxidase complex described in any one of claims 1 - 6, which polypeptide contains an amino acid sequence identified by SEQ ID NO: 4, or an amino acid sequence having 85 % or higher identity  
15 with the said amino acid sequence and is capable of providing the said complex with cytochrome c oxidase activity.

10. A recombinant polypeptide as set forth in claim 9 capable of providing the complex described in any one of claims 1 - 6 with cytochrome c oxidase activity, which is encoded by a recombinant DNA fragment containing a DNA sequence selected from the  
20 group consisting of:

- (a) the DNA sequence identified by SEQ ID NO: 3, and
- (b) the DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 4 or amino acid sequences having 85 % or higher identity with the said amino acid sequence.

25 11. A recombinant polypeptide which is involved in cytochrome c oxidase complex described in any one of claims 1 - 6, which polypeptide contains an amino acid sequence identified by SEQ ID NO: 6 or 8, or an amino acid sequence having 85 % or higher identity with either of the said amino acid sequences of SEQ ID NOs: 6 and 8 and is capable of providing the said complex with cytochrome c oxidase activity.

30 12. A recombinant polypeptide as set forth in claim 11 capable of providing the complex described in any one of claims 1 - 6 with cytochrome c oxidase activity, which is encoded by a recombinant DNA fragment containing a DNA sequence selected from the group consisting of:

- (a) the DNA sequence identified by SEQ ID NO: 5,
- 35 (b) the DNA sequence identified by SEQ ID NO: 7,

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(c) the DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 6 or amino acid sequences having 85 % or higher identity with the said amino acid sequence, and

(d) the DNA sequences which encode polypeptides having an amino acid sequence  
5 identified by SEQ ID NO: 8 or amino acid sequences having 85 % or higher identity with the said amino acid sequence.

13. A recombinant DNA fragment, which comprises a DNA sequence claimed in claim 8 and which encodes an amino acid sequence of a polypeptide capable of providing the complex described in any one of claims 1 - 6 with cytochrome c oxidase activity,  
10 wherein the said DNA sequence is selected from the group consisting of:

(a) the DNA sequence identified by SEQ ID NO: 1, and

(b) the DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 2 or amino acid sequences having 85 % or higher identity with the said amino acid sequence.

15 14. A recombinant DNA fragment, which comprises a DNA sequence claimed in claim 10 and which encodes an amino acid sequence contained in a polypeptide capable of providing the complex described in any one of claims 1 - 6 with cytochrome c oxidase activity, wherein the said DNA sequence is selected from the group consisting of:

(a) the DNA sequence identified by SEQ ID NO: 3, and

20 (b) the DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 4 or amino acid sequences having 85 % or higher identity with the said amino acid sequence.

15 15. A recombinant DNA fragment, which comprises a DNA sequence claimed in claim 12 and which encodes an amino acid sequence contained in a polypeptide capable of providing the complex described in any one of claims 1 - 6 with cytochrome c oxidase activity, wherein the said DNA sequence is selected from the group consisting of:

(a) the DNA sequence identified by SEQ ID NO: 5,

(b) the DNA sequence identified by SEQ ID NO: 7,

30 (c) the DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 6 or amino acid sequences having 85 % or higher identity with the said amino acid sequence, and

(d) the DNA sequences which encode polypeptides having an amino acid sequence identified by SEQ ID NO: 8 or amino acid sequences having 85 % or higher identity with the said amino acid sequence.

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16. An expression vector comprising one or more recombinant DNA fragments selected from the fragments claimed in claims 13, 14, and 15 wherein the expression vector is suitable for expression in an organism.

17. An expression vector according to claim 16, wherein the organism is a  
5 microorganism selected from the group consisting of bacteria such as *Escherichia coli*, *Pseudomonas putida*, *Acetobacter xylinum*, *Acetobacter pasteurianus*, *Acetobacter aceti*, *Acetobacter hansenii* and *Gluconobacter oxydans*.

18. An expression vector according to claim 16, wherein the organism is a  
10 microorganism identified as *Gluconobacter oxydans* DSM 4025 or biologically and/or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of *Gluconobacter oxydans* DSM 4025.

19. A recombinant organism which is introduced by an expression vector claimed in any one of claims 16 - 18.

20. A recombinant organism as claimed in claim 18, in which the recombinant  
15 organism carries one or more DNA fragments selected from those claimed in claim 13, 14 and 15 integrated into its genome.

21. A recombinant organism according to claim 19 or 20, wherein a host organism is a microorganism selected from the group consisting of bacteria such as *Escherichia coli*, *Pseudomonas putida*, *Acetobacter xylinum*, *Acetobacter pasteurianus*, *Acetobacter aceti*,  
20 *Acetobacter hansenii* and *Gluconobacter oxydans*.

22. A recombinant organism according to claim 19 or 20, wherein a host organism is a microorganism identified as *Gluconobacter oxydans* DSM 4025 or biologically and/or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of *Gluconobacter oxydans* DSM 4025.

23. A process for producing cytochrome c oxidase complex, which comprises  
25 cultivating a recombinant organism defined in any one of claims 19 - 22 in an appropriate culture medium and recovering the cytochrome c oxidase from the culture.

24. A process according to claim 23, wherein a host organism is a microorganism selected from the group consisting of bacteria such as *Escherichia coli*, *Pseudomonas*  
30 *putida*, *Acetobacter xylinum*, *Acetobacter pasteurianus*, *Acetobacter aceti*, *Acetobacter hansenii* and *Gluconobacter oxydans*.

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25. A process according to claim 23, wherein a host organism is a microorganism identified as *Gluconobacter oxydans* DSM 4025 or biologically and/or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of *Gluconobacter oxydans* DSM 4025.

5        26. A process for producing 2-keto-L-gulonic acid from L-sorbose or D-sorbitol, which comprises cultivating a recombinant organism defined in any one of claims 19 - 22 in an appropriate culture medium and recovering 2-keto-L-gulonic acid from the culture.

10       27. A process according to claim 26, wherein a host organism is a microorganism selected from the group consisting of bacteria such as *Escherichia coli*, *Pseudomonas putida*, *Acetobacter xylinum*, *Acetobacter pasteurianus*, *Acetobacter aceti*, *Acetobacter hansenii* and *Gluconobacter oxydans*.

15       28. A process according to claim 26, wherein a host organism is a microorganism identified as *Gluconobacter oxydans* DSM 4025 or biologically and/or taxonomically homogeneous cultures of a microorganism having the identifying characteristics of *Gluconobacter oxydans* DSM 4025.



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17. Nov. 1999

Abstract

The present invention is directed to a novel cytochrome c oxidase complex, genetic materials useful for the preparation of the said complex, such as recombinant polypeptides  
5 involved in cytochrome c oxidase complex, recombinant DNA fragments, expression vectors, recombinant organisms and the like. Those novel cytochrome c oxidase complex and genetic materials may be originated from a microorganism having the identifying characteristics of *Gluconobacter oxydans* DSM 4025. The present invention also provides a method for the preparation of the said novel recombinant cytochrome c oxidase complex  
10 and a process for the production of 2-keto-L-gulonic acid (2KGA).

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17. Nov. 1999

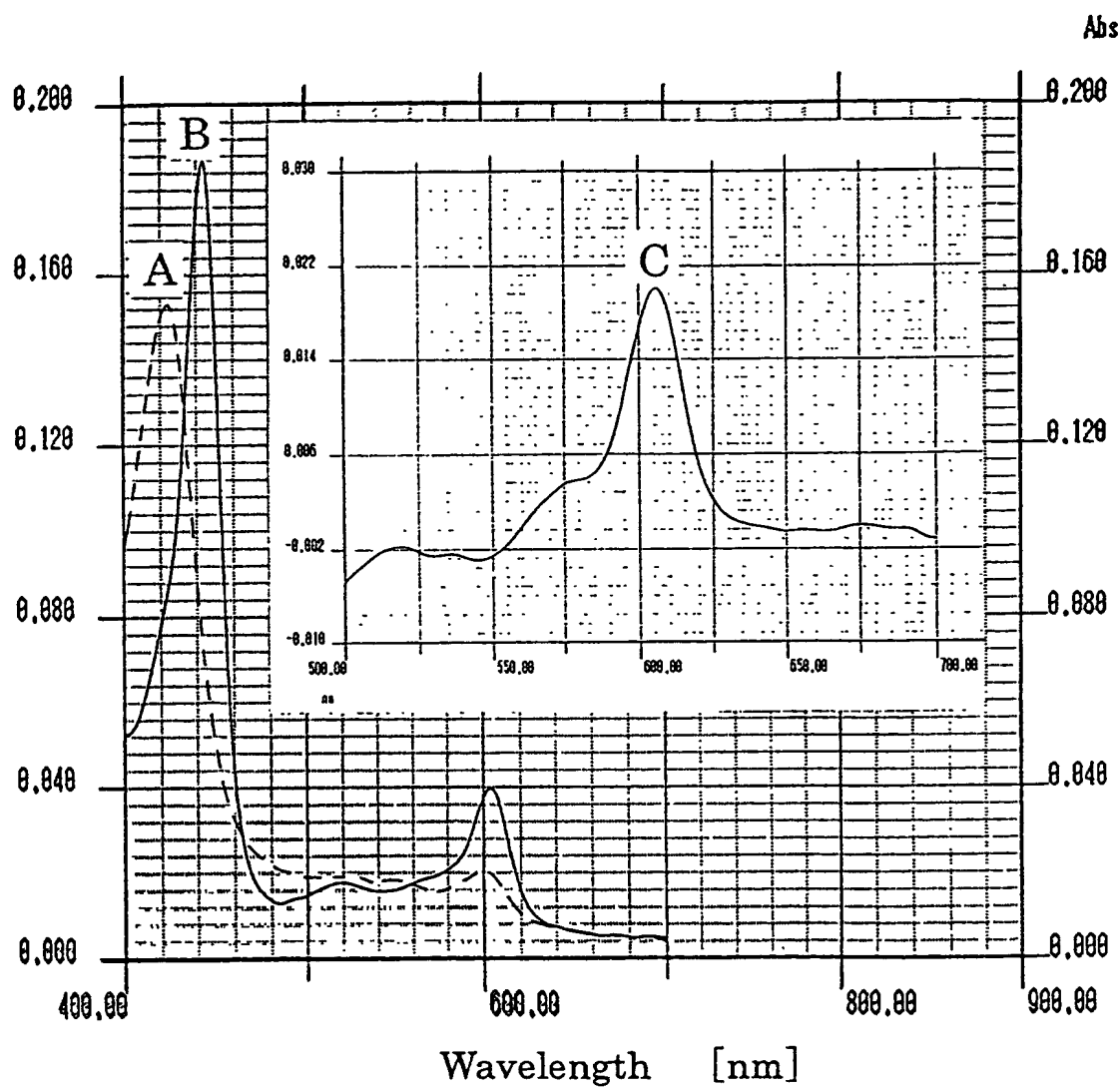


Figure 1

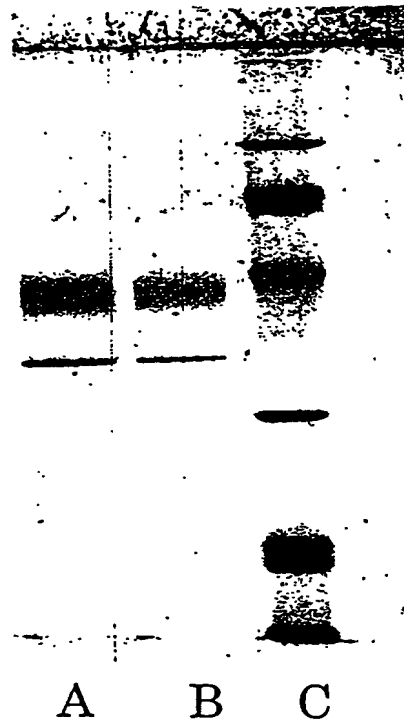


Figure 2

## CO I

**G.O./peptide** **KDIGLLYLVAAGVVG**

P.D. 1:MS-AQISDSIEEKRGFFTRWFMSTN HKDIGVLYLFTAGLAGLISVTLTVYMRMELQHPGVQYMC---LE-  
R.S. 1:MADAAIHGHEHRRGFFTRWFMSTN HKDIGVLYLFTGGLVGLISVFTVYMRMELMAPGVQFMCAEHLES  
B.M. 1:-----M-FINRWLFSTN HKDIGTLYLLFGAWAGMVG---TA-LSL-L----IR---AE-L--

P.D. 71:GM-R-LVAD--AAA-E-CTPNAHLWNVVV TY-HGILMMFFVVIPALFGGFGNYFMPLHIGAPDMAFPRLN  
R.S. 71:GLVKGFFQSLWPSAVENCTPNGLWNVMI-YGHGILMMFFVVIPALFGGFGNYFMPLHIGAPDMAFPRLN  
B.M. 71:GQ-PG---TL----LGD---D-QIYNVVVT-AHAFVMIFFMVMPIMIGGFGNWLVPMLIGAPDMAFPRLN

P.D. 141:NLSYWL YCGVSLAIASLLSPGGS DQPGAGVGWVLYPPL-ST-TEAGYAMD LAIFAVH VSGATSILGAIN  
R.S. 141:NLSYWL YAGTSLAVASL FAPGGNGQLGSGIGWVLYPPL-ST-SESGYSTDLAIFAVH LSGASSILGAIN  
B.M. 141:NMSFWLLPPSFL LLLASSMVEAG---AGT---GWTVPPLAGNLAHAGASVDLTIFSLHLAGVSSILGAIN

P.D. 211:IITTF LNM RAPGMTL FKVPLFAWAVF ITAWMILLSLPVL AGGITM LMDRNF GTQFFD PAGGGDPVLYQH  
R.S. 211:MITTF LNM RAPGMTM HKVPLFAWSIFVTAWL ILLALPVL AGAITM LLDNRNF GTTFFQPSGGGDPVLYQH  
B.M. 211:FITTI INMKPPAMSQYQTPLFVWSVMITAVL LLLSLPVL AAGITM LLDNRNLNTFFDPAGGGDPILYQH

**G.O./PCR** **WFFGHPEVYIIILPGFGIISHVSTFS-KKPVFGYLPVYAMLAIGVLGFVVAHMM**

>>>>>> <<<<<<<

P.D. 281:ILWFFGHPEVYMLILPGFGIISHVISTF-ARKPIFGYLPVYAMLAIAFLGFIVWAHMYTAGMSLTQQT  
R.S. 281:ILWFFGHPEVYIIIVLPAFGIVSHVIATF-AKKPIFGYLPVYAMVAIGVLGFVVAHMYTAGLSLTQQS  
B.M. 281:LFWFFGHPEVYIILPGFGMISHIVTYYSKGKEPPFGYMGVWAMMSIGFLGFIVWAHMYTVGMDVDTRA

P.D. 351:YFQMATMTIAVPTGIKVF SWIATMGGSI EFKTPMLWALA--FLFTVGGVTGVVIAQGS LDRVYHDTYYI  
R.S. 351:YFMMATMVIAPVTGIKIF SWIATMGGSI ELKTPMLWALGFLFLFTVGGVTGIVLSQASVDRYYHDTYYV  
B.M. 351:YFTSATMIIAIP TGVKVFSWLATLHG GNIKWSPAMMWALGFI FLFTVGGLTGIVLANSSLDIVLHDTYYV

P.D. 421:VAHFHYVMSLGALFAIFAGTYYSIGKMSGRQYPE-WAGQLHFWMF IGSNLIFFPQHFLGRQGMPPRYID  
R.S. 421:VAHFHYVMSLGAVFGIFAGSTSGIGKMSGRQYPE-WAGKLHFWMFVGANLTFFPQHFLGRQGMPPRYID  
B.M. 421:VAHFHYVLSMGAVFAIMGGFVHWFPLFSGYTLNDTWA-KIHFAIMFVGVMNTFFPQHFLGLSGMPPRYSD

P.D. 491:YPVEFSYWN NISSIGAYISFASFLFFIGIVFYTLFAGKPVNVPNYWNEHADTLEWTLSPPPPEHTFETLP  
R.S. 491:YPEAFATWNFVSSLGAFLSFASFLFFLGVIIFYSL-SGARVTANNYWNEHADTLEWTLSPPPPEHTFEQLP  
B.M. 491:YPDAYTMWNTISSMGSFISLTAVMLMV-FIIWEAFASKR-EVLTVDLTITNLEWLNGC PPHYHTFEE-P

P.D. 561:KPEDWDRAQ AHR  
R.S. 561:KREDWERAPAH-  
B.M. 561:TYVN-LK-----

Abbreviations: G.O. *G. oxydans* DSM4025; P.D. *Paracoccus denitrificans*;  
R.S. *Rhodobacter sphaeroides*; B.M. Bovine (Mitochondria).

>>>>>> <<<<<<< : amino acid sequences for PCR primers (Fig. 6)

G.O./peptide: amino acid sequence from the purified enzyme

G.O./PCR: amino acid sequence deduced from DNA amplified by PCR (PCR/DNA)

Figure 3

## COII

P.D. 1:MAIATKRRGVAAVMSLG VATMTAVPALAQDVLGDLFVIGKPVNGGMNFQPASSPLAHDQQWLDHFVLYII  
 R.S. 1:MRHSTLTTPCATGAA-GLLAATAAAA-QQOTL-E--IIIGRPQGGTGFGHSASP VATQIH WLDGFILVII  
 B.M. 1:-----MAYPM-Q--LG-FQDATSPIM--EELLHFHD-HT---L-MIVF-L--I

G.O./peptide KASQFTHNTPLEIVWTIVPV  
 G.O./PCR QFTHNTPLEIVWTIVPVVILVFIFGAFSLPVLFKQQEFPE-GDI  
 >>>>>

P.D. 71:TAVTIFVCLLLLICIVRFNRRANPVPARFTHNTPIEVIWTLVPVLILVAIGAFSLPILFRSQEMPNDPDL  
 R.S. 71:CAITIFVTLILYAVWRFHEKRNKVPARFTHNSPLEIAWTIVPIVILVAIGAFSLPVLFNQEIPE-EADE  
 B.M. 71:SSLVLYIISLIL--TTKL/THTSTMDP----QE--VETIWTILPAIILILIALPSLRILYMMDEI-NNPSL

G.O./PCR VINVEGRSWYWGYE  
 <<<<<<

P.D. 141:VKAIGHQWYWSYEPNDAFAFDALML-----E-K-----E-ALADAGYSEDEYLLATDNFVVVPVG  
 R.S. 141:TVKVTGYQWYWGYPDEEISFESYMIGSPATGGDNRM-SPEVEQQLIEAGYTRDEFLATDTAMVVPVN  
 B.M. 141:TVKTMGHQWYWSYEY----TDY-EDL--S--L-D-SYM-IPTSE--L-KPG---ELRLLEVDNRVVLPM

P.D. 211:KKVLVQVTATDVIHAWTIPAFVAVKQDAVPGRIAQLWFSVDQEGVYFGQCSELCGINHAYMPIVVKAVSQE  
 R.S. 211:KTVVVQVTGADVHSWIVP-FGVRQDAVPGRLAQLWFAEREGIFFGQCSELCGISHAYMPIVVKVSEE  
 B.M. 211:MTIRMLVSSGDVLHSAVPSLGLKTDAPGRNLQTTLMSSRPGLYYGQCSEICGSNHSFMPIVLELVPLK

P.D. 281:KYEAWLAGAKEEFAADASDYLPA SPVKLASAE  
 R.S. 281:AYAAWLEQARGG-TYELSSVLPATPAGV-SVE  
 B.M. 281:YFEKW-SASML-----

Abbreviations: G.O. *G. oxydans* DSM4025; P.D. *Paracoccus denitrificans*;

R.S. *Rhodobacter sphaeroides*; B.M. Bovine (Mitochondria).

>>>>> <<<<<< : amino acid sequences for PCR primers (Fig. 6)

G.O./peptide: amino acid sequence from the purified enzyme

G.O./PCR: amino acid sequence deduced from DNA amplified by PCR (PCR/DNA)

Figure 4

## CO III

P.D. 1:MAHVKNHDYQILPPSIWPFPGAIGAFVMLTGAVAWMKGITFFGLPVEGPWMFLIGLVGVLYVMFGWWADV  
 R.S. 1:MAHAKNHDYHILPPSIWPFMASVGAFVMLNGAVLWMH-----G-S--GPWMGLIGLVVLYTMFGWWSOV  
 B.M. 1:MTH-QTHAYHMVNPSWPPLTGALSALLMTSGLTMW---FHFSMTL-----L-MIGLTTNMLTMYQWWRDV

P.D. 71:VNE-GETGEHTPVVRIGLQYGFILFIMSEVMFFVAVFWAFIKNALYPMGPDSPKDGVMPPGIVTFDPW  
 R.S. 71:VTE-SLEGDHTPVVRLGLRWGFILFIMSEVIFFSAWFWSFFKHALYPMGPESPIIDGIFPPEGIITFDPW  
 B.M. 71:IRESTFQGHHTPAVQKGLRYGMILFIISEVLFFTGFVAFYHSSL---AP-TPELGCCWPPTGIHPLNPL

G.O./PCR TWAHHA-IVHGDRKKTATGLAIAIGLWIFTLCQAYEYETVHTE:xxxxxxx  
 >>>>>

P.D. 141:HLPLINTLILLSGVAVTWAHHAFVLEGDRKTTINGLIVAVILGVCFTGLQAYEYSHAAGLADTVYAG  
 R.S. 141:HLPLINTLILLCSGCAATWAHHALVHNNRRDVAWGLALALGALFTVFQAYEYSHAAGFAGTIYGA  
 B.M. 141:EVPLLNTSVLLASGVSITWAHHS-LMEGDRKMLQALFITITLGVYFTLLQASEYEAFTISDGVYGS

G.O./PCR xxxxxxxxxxxxxxxD-SIFLLVCLIRILRGAMSAKQHVGFEMAANYWHFV

<<<<<<

P.D. 211:AFYMATGFHGAHVIIIGTIFLVCLIRLLKGAMTQKQHVGFEEAAWYWHFVDVWVLFVVIYIWGR  
 R.S. 211:NFFMATGFHGFHVITVGTIFLLVCLIRVQRGHFTPEKHVGFEEAMWYWHFVDVWVLFVSIYIWGQ  
 B.M. 211:TFFVATGFHGLHVIIGSTFLIVCFRQLKFHFTSNHHFGFEAGAWYWHFVDVWVLFVSIYIWGS

Abbreviations: G.O. *G. oxydans* DSM4025; P.D. *Paracoccus denitrificans*;  
 R.S. *Rhodobacter sphaeroides*; B.M. Bovine (Mitochondria).

>>>>> <<<<<< : amino acid sequences for PCR primers (Fig. 6)

G.O./PCR: amino acid sequence deduced from DNA amplified by PCR (PCR/DNA)

Figure 5

# CO I (target size: approx. 180 bp DNA)

A:                   W F F G H P                   →                   \*

5'-TGGTTCTTCGGNCACCC-3'

          T T T

B:                   ←                   V W A H H M                   \*

3'-CANACCCGNGTAGTATAC-5'

                  G G

# CO II (target size: approx. 180 bp DNA)

A:                   Q F T H N T                   →                   \*\*

5'-CAATTTACNCATAATAC-3'

          G C C C

B:                   ←                   W Y W G Y E Y                   \*

3'-ACCATAACCCCNATACTTAT-5'

                  G G C

# CO III (target size: approx. 300 bp DNA)

A:                   T W A H H A                   →                   \*

5'-CANTGGGCNCATCATGC-3'

                  C C

B:                   ←                   W Y W H F V D                   \*

3'-ACCATAACCGTAAAACANCT-5'

                  G G G

N : A, T, G, C

\* : based on consensus sequence

\*\* : based on the peptide sequence

Figure 6

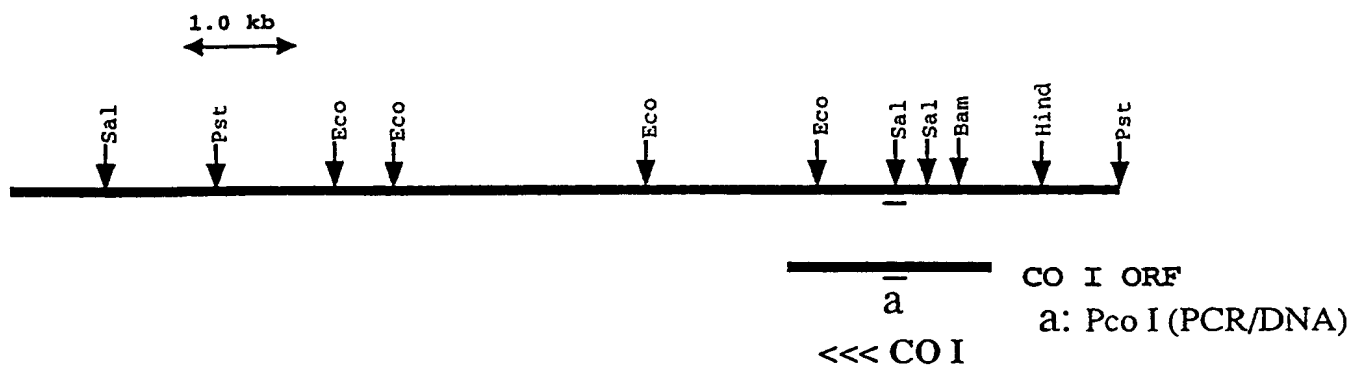
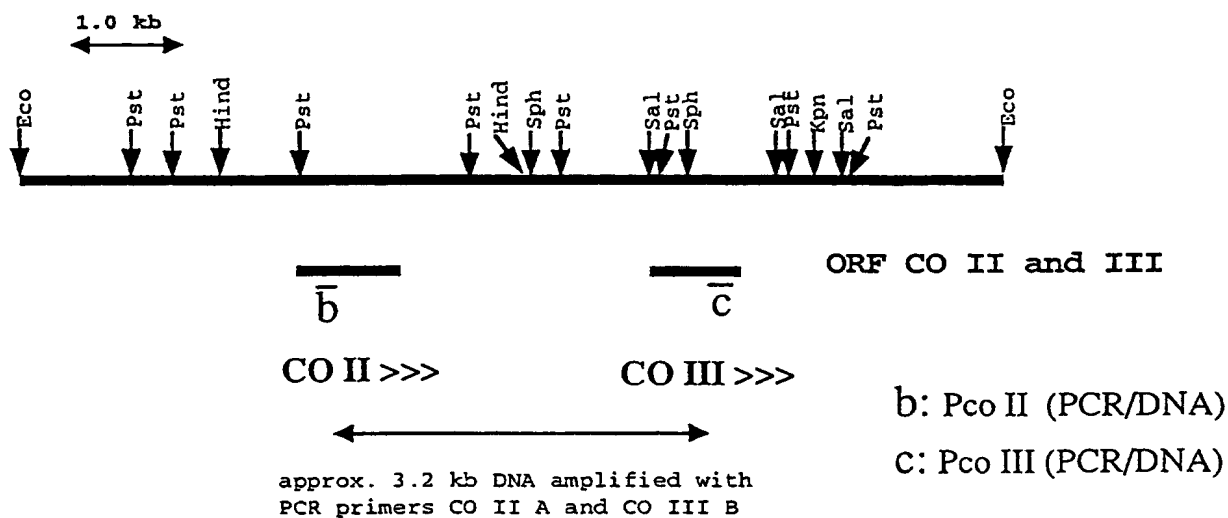
8.0 kb *Pst*I fragment containing CO I ORF of pUCO019.3 kb *Eco*RI fragment containing CO II and III ORFs of pUCO23

Figure 7

B.M. : -----M-FINRWLFSTNHKDIGTLYLLFGAWAGMVGTALSLLIRAE LGQPG--TL-----L-----G--D-  
 P.D. : MS-AQI-SDSIEEKGFFTRWFMSTNHKDIGVLYLFTAGLAGLISVTLTVYMRMELQHPGVQYMC---LE-GM-R-LV-A  
 R.S. : MADAIIHGHE-HDRRGFFTRWFMSTNHKDIGVLYLFTGGLVGLISVAFVYMRMELMAPGVQFMCAEHLESGLVKGFFQS  
 G.O. : MADAIIHGHDHHEKQGFTRWFMSTNHKDIGLLYLVAAGVVGFI SVLFTVYMRLELMDPGVQYMC---LE-G-AR--L--

B.M. : -----D-QIYNVVVT-AHAFVMIFFMVMPIMIGGFGNWLVLPMIGAPDMAFPRMNNMSFWLLPSPFLLLLASSM  
 P.D. : ---DAAAECTPNAHLWNVVVY-HGILMMFFVVI PALFGGFGNYFMP LHI GAPDMAFPRMNNLSYWLVCVSLAIASLL  
 R.S. : LWPSAVENCTPNGLWNVMI-YGHGILMMFFVVI PALFGGFGNYFMP LHI GAPDMAFPRMNNLSYWLVCVSLAVASLF  
 G.O. : I-ADASQTCTANGHLWNVMVY-HGILMMFFVGI PALFGGFGNYLMP LQI GAPDMAFPRMNNLSFWLFIAGTAMGVASLF

B.M. : VEAG---AGT--GWTVPPLAGNLAHAGASVDLTIFSLHLAGVSSILGAINFITTI INMKPPAMSQYQTP LFVWSVMITA  
 P.D. : SPGGSDQPGAGVGVWLYPPL-ST-TEAGYAMD LAIFAVHVS GATSILGAINIITTF LNM RAPGMTLFKVPLFAWAVFITA  
 R.S. : APGGNGQLGSGIGVWLYPPL-ST-SESGYSTDLAIFAVHLSGASSILGAINMITTF LNM RAPGMTMHKVPLFAWSIFVTA  
 G.O. : APGGGQQLGSGVGVWLYPPL-ST-REAGYSMDLAIFAVHLSGASSIMGAINMITTF LNM RAPGMTLHKVPLFWSIFITA

B.M. : VLLLLSLPVLAAITMLLTDRNLNTTFFDPAGGGDPILYQHLWFFGHPEVYIILPGFGMISHIVTYYSKKKEPFGYMG  
 P.D. : WMILLSLPVLAGGITMLLMDRNFQTQFFDPAGGGDPVLYQHILWFFGHPEVYMLILPGFGIISHVISTF-ARKPIFGYLP  
 R.S. : WLILLALPVLAGAITMLLTDRNFGTTFQPSGGGDPVLYQHILWFFGHPEVYIIVLPAFGIVSHVIATF-AKKPIFGYLP  
 G.O. : WLILLALPVLAGAITMLLTDRNFGTTFPNPAGGGDPILYQHILWFFGHPEVYIILPGFGIISHVVSTF-SKKPVFGYLP

B.M. : MVWAMMSIGFLGFIWVAHMHFTVGMDDVTRAYFTSATMIIAIP TVKVFSWLATLHGGNIKWSPAMMWALGFIFLFTVGG  
 P.D. : MVLAMAAIAFLGFIWVAHMHYTAGMSLTQQTYPQMATMTIAVPTGIKVS WIATMWGGSIEFKTPMLWA--LAF LFTVGG  
 R.S. : MVYAMVAIGVLGFVVAHMHYTAGLSLTQQSYFMMATMNI AVPTGIKIFSWIATMWGGSIELKTPMLWALGF LFTVGG  
 G.O. : MVYAMVAIGVLGFVVAHMHYTVGMSLTQQSYFMLATMVI AVPTGIKIFSWIATMWGGSVEFKSPMLWAFGMFLFTVGG

B.M. : LTGIVLANSSLDIVLHDTYYVVAHFHYVLSMGAVFAIMGGFVHWFLFSGYTLNDTWA-KIHFAIMFVGVNMTFFPQHFL  
 P.D. : VTGVVIAQGS LDRVYHDTYYIVAHFHYVMSLGALFAIFAGTYYSIGKMSG-RQYPEWAGQLHFWMF IGSNLIFFPQHFL  
 R.S. : VTGIVLSQASVDRYHDTYYVVAHFHYVMSLGAVFGIFAGSTSGIGKMSG-RQYPEWAGKLHFWMFVGANLTFPPQHFL  
 G.O. : VTGIVLAQAGLDRAYHDTYYVVAHFHYVMSLGALFAIFAGIYFYMPKFSG-RAFPEWAAKLHFWTFFIGANVTFFPQHFL

B.M. : GLSGMPRRYSDYDAYTMWNTISSMGSFISLTAVM-L--MVFIWEAFASKREVLTV-DLTTTNLEWLNCGPPPYHTFEE  
 P.D. : GRQGMPPRYIDYPVEFSYWNMISSIGAYISFASLFFIGIVF-YTLFAGKPVNPNYWNEHADTLEWTLSPPPPEHTFET  
 R.S. : GRQGMPPRYIDYPEAFATWNFVSSLGAFSLFASFLFLGVIF-YSL-SGARVTANNYWNEHADTLEWTLSPPPPEHTFEQ  
 G.O. : GRQGMPPRYIDYPEAFALWNKVSSYGAFSLFASFLFFI-VIFVYTLVAGRRRETRPNPWGEFADTLEWTLSPPPPEHTFET

B.M. : -PTYVN-LK-----  
 P.D. : LPKPEDWDRAQAH  
 R.S. : LPKREDWERAPAH-  
 G.O. : LPKRSDWDKHPSH-

Homology among amino acid sequences of the CO I subunits of  
 aa3-type cytochrome c oxidase

	<u>B.M.</u>	<u>P.D.</u>	<u>R.S.</u>
<u>P.D.</u>	52.2	-	-
<u>R.S.</u>	50.6	76.7	-
<u>G.O.</u>	53.3	76.0	78.7

[% homology]

Abbreviations: G.O. *G. oxydans* DSM4025; P.D. *Paracoccus denitrificans*;  
 R.S. *Rhodobacter sphaeroides*; B.M. Bovine (Mitochondria)

Figure 8



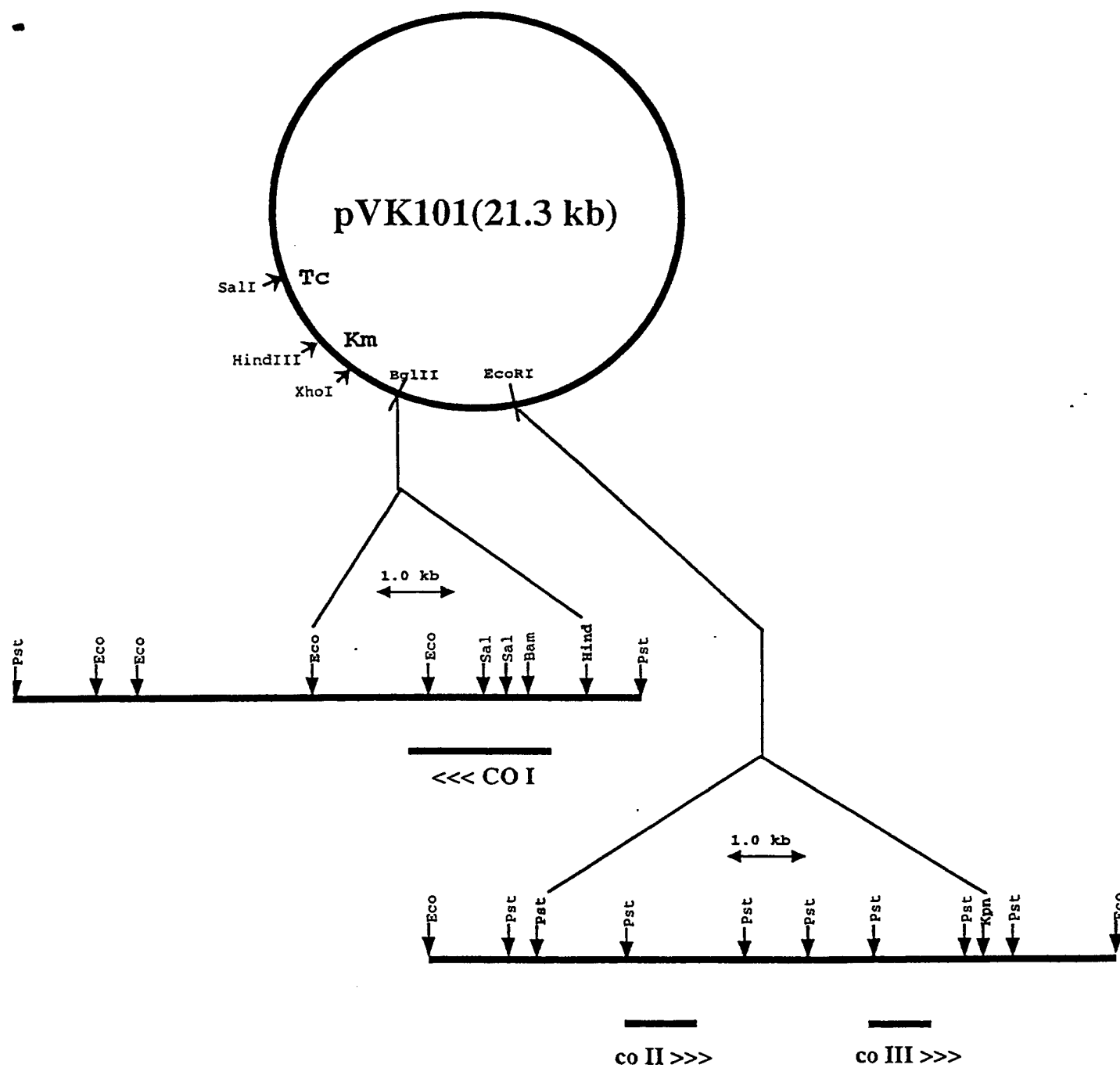


Figure 9

